MOLECULAR DETERMINANTS OF COLLYBISTIN FUNCTION IN GABAERGIC SYNAPSES: *IN VITRO* AND *IN VIVO* ANALYSIS

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ZUSAMMENFASSUNG

Collybistin (CB) ist ein Neuronen-spezifischer GDP/GTP Austauscher aus der dbl-Familie, welcher selektiv die kleinen RhoGTPasen Cdc42 und TC10 aktiviert. CB wird von einem einzigen Gen kodiert, welches mittels alternativem Spleissen drei CB Isoformen (CB1-CB3) hervorbringt. Der Unterschied besteht hauptsächlich in der Sequenz und Länge des C'-terminus und dem Vorhandensein einer SH3 Domäne. Die Interaktion von CB mit Gephyrin, dem wichtigsten Strukturprotein in GABAergen und glycinergen Synapsen, ermöglicht das Clustern und Stabilisieren von Gephyrin in GABAergen Synapsen und dadurch die Bildung und Plastizität der GABAergen Synapsen. GABAerge Synapsen sind sehr dynamisch und passen ihre Stärke und Lokalisierung den globalen und lokalen Veränderungen der Netzwerk Aktivität an, um das Gleichgewicht zwischen Exzitation und Inhibition aufrecht zu erhalten. Um dies zu erreichen, treffen verschiedene Signal-Kaskaden in der postsynaptischen Verdichtung (PSD) zusammen und modulieren dadurch die Gephyrin-Cluster-Bildung und das Actin Zellskelet. Obwohl mehrere Studien auf die Natur von CB und Gephyrin Interaktion an der Synapse eingegangen sind, blieben sowohl die exakte Rolle der verschiedenen Spleissisoformen sowie die Relevanz der Cdc42-Aktivierung unerforscht. Es ist vorstellbar, dass CB ein Multifunktionales Protein ist, das sowohl Gephyrin-Cluster Bildung in der PSD reguliert, als auch das Zellskelet durch Cdc42-Aktivierung verändert. Diese Dissertation hatte das für Ziel, die Relevanz von CB Isoformen für die GABAerge synaptische Plastizität zu erforschen, mit dem Fokus auf post-transcriptionale und post-translationale Regulierung in kultivierten Neuronen. Des Weiteren erforschten wir in einem *in vivo* Model den Effekt von CB-Überexpression und -Unterdrückung auf die Reifung und synaptische Integration von neugeborenen Neuronen im erwachsenen Gehirn, um die Bedeutung einer Cdc42-Aktivierung durch CB hervorzuheben.

Die Ergebnisse der *in vitro* Studie zeigten, dass CB distal von $GABA_AR$ nachgeschaltet wird um Gephyrin in die Synapse zu rekrutieren. Des Weitern fanden wir eine Aktivitäts-abhängige Regulierung der Wahl der Spleissstelle für CB, welche über eine Aktivierung der CaMKII/IV Signalisierung die CB1 Expression reduziert. Auf der post-translationalen Ebene konnten wir bestimmen, dass die Halbwertszeiten der

CB Isoformen deutlich unterschiedlich sind, und dass sie durch die Ubiquitinierung von Lysinen in den Isoform-spezifischen C'-terminalen Domänen von CB bestimmt werden. Durch Überexpression von stabilen CB Mutanten konnten wir beweisen, dass eine direkte Verbindung zwischen CB-Stabilität und Gephyrin-Clustern in der PSD besteht. Zusammengefasst, deuten unsere Befunde darauf hin, dass CB2 eine stabile Isoform ist, unempfindlich gegen Aktivität-bedingte Veränderungen, und dass CB1 dynamisch reguliert wird. Schliesslich konnten wir mittels einem empfindlichen *in situ* Hybridisierungs-Verfahren mRNA Molekülen in Dendriten detektieren, welche eine lokale Proteinsynthese annehmen lässt und folglich, dass die unterschiedlichen Halbwertszeiten der CB Isoformen einer Synapsen-spezifischer Regulation der GABAergen Übertragung unterliegen, vermittelt durch CB-Gephyrin Interaktionen.

In der *in vivo* Studie diente die Adulte Neurogenese als Modelsystem für die Untersuchung der Wirkung von CB auf die Reifung und die Bildung von GABAergen Synapsen in neugeborenen Neuronen im Gyrus Dentatus. Mittels intra-hippocampaler Retrovirusinjektion wurden selektiv mitotische neuronale Vorläuferzellen infiziert, entweder mit GFP-markiertem CB für die Überexpression, oder mit GFP zusammen mit shRNA Konstrukten um CB Expression zu unterdrücken, wobei die Zelle dank des GFP-Reporters markiert wird. Überraschenderweise führte sowohl die Überexpression wie auch die Unterdrückung von CB in wildtyp Mäusen zu einer dendritischen Hypertrophie, verbunden mit einer gestörten Migration, was einer konstitutiven Aktivierung von Cdc42 zugeschrieben werden kann. Die Analyse der postsynaptischen Cluster von α1- und α2-$GABA_A$R-Untereinheiten zwischen 14 und 42 Tagen nach der Injektion (dpi) zeigte keine grösseren Unterschiede unter beiden Bedingungen; diese Beobachtung liegt im Widerspruch mit den Resultaten von kultivierten Neuronen. In α2-KO Mäusen, hingegen, führte die Überexpression von CB zu einer Wiederherstellung von Gephyrin-Clustern und einer transienten Erhöhung von α1-Untereinheit-Clustern, welche möglicherweise das Fehlen von α2-$GABA_A$R kompensiert. Diese Ergebnisse deuten an, dass die Aktivierung von Cdc42 durch CB unter normalen Bedingungen streng reguliert wird, möglicherweise durch eine Konformationsänderung, welche von der Bindung mit Gephyrin herbeigeführt wird und die enzymatische Aktivität von CB aktiviert. Einem solchen Model zufolge,

wäre CB ein Schlüsselregulator von Cdc42, nicht nur für die Regulierung von GABAerger Synapsen Bildung und Plastizität, sondern auch für die morphologische Differenzierung von Neuronen während der Entwicklung.

Diese beiden Studien enthüllen zwei unterschiedliche Funktionen von CB: Isoform-spezifische Regulation von Gephyrin-Cluster-Bildung um GABAerge synaptische Plastizität zu fördern, und die Regulierung der Cdc42-Aktivität während der Reifung und Entwicklung von Neuronen, in Abhängigkeit von der Bindung mit Gephyrin und synaptischer Lokalisation.

ABSTRACT

Collybistin (CB) is a neuron-specific GDP/GTP-exchange factor (GEF) of the dbl family, selectively activating the small Rho GTPases Cdc42 and TC10. It is encoded by a single gene, but alternatively spliced into three isoforms (CB1-CB3), differing mainly in their C'-terminal domain and by the presence/absence of an N-terminal SH3 domain. CB interacts with gephyrin, the major scaffolding protein at GABAergic and glycinergic synapses. CB is essential for gephyrin clustering and stabilization at GABAergic synapses, thus contributing to their formation and plasticity. GABAergic synapses are highly dynamic and adapt their strength and location to global and local changes in network activity, in order to maintain the balance between excitation and inhibition. To this end, multiple signaling cascades converge at the post-synaptic density (PSD) and modulate gephyrin clustering and the actin cytoskeleton. Although several studies focused on the nature of CB/gephyrin interaction at the synapse, the precise role of the different splice isoforms and the importance of Cdc42 activation remained unexplored. It is conceivable that CB is a multifunctional protein, regulating both gephyrin clustering at the PSD and modifying the cytoskeleton through Cdc42 activation. The present thesis was designed to explore the relevance of CB isoforms for the modulation of GABAergic synaptic plasticity. The research focused on different post-transcriptional and post-translational regulatory mechanisms *in vitro*. Further, using an *in vivo* system, we investigated the effects of CB overexpression and down-regulation on the maturation and synaptic integration of newborn neurons in adult brain in order to uncover the significance of CB-mediated Cdc42 activation.

The results of the *in vitro* study revealed that CB acts downstream of $GABA_A$Rs to recruit gephyrin to the synapse. Furthermore, we uncovered activity-dependent regulation of CB splice-site selection, reducing CB1 expression by a mechanism involving CaMKII/IV signaling. At a post-translational level, we report differential turnover of CB isoforms, determined by ubiquitination of isoform-specific lysine residues in the C'-terminal domain. By overexpressing stable CB mutant constructs, we demonstrate increased impact on gephyrin postsynaptic clustering. Taken together, our findings suggest that CB2 is a stable isoform, insensitive to activity

changes, whereas CB1 is dynamically regulated. Finally, we observed the presence of CB mRNA in dendrites, suggesting that local protein synthesis and differential turnover of isoforms underlie synapse-specific regulation of GABAergic transmission via isoform-dependent CB-gephyrin interactions.

The *in vivo* study used adult neurogenesis as a model system to investigate the effects of CB on maturation and GABAergic synapse formation of newborn neurons in the dentate gyrus. Through retroviral intra-hippocampal injections, we infected only dividing neurons with either GFP-tagged CB to overexpress CB or GFP and shRNA constructs to silence CB expression while labeling the transfected cells. Unexpectedly, the overexpression and the silencing of CB in wild type mice both resulted in dendritic hypertrophy together with impaired migration, suggesting a constitutive activation of Cdc42. The analysis of $GABA_AR$ α1 and α2 subunit postsynaptic cluster density between 14 and 42 days post-injection did not reveal major effects in either condition, which is opposite to the findings from cultured neurons. However, CB overexpression could restore gephyrin clustering in α2-KO mice and transiently increase α1 subunit clustering, possibly compensating for the lack of α2-$GABA_AR$. These results suggest that the activation of Cdc42 by CB is tightly regulated under normal conditions, possibly via post-translational regulatory mechanism(s) and/or via interaction with gephyrin that would induce a conformational change required for its enzymatic activity. According to such a model, CB would be a key regulator of Cdc42 for modulating not only GABAergic synapse formation and plasticity, but also morphological differentiation of neurons during development.

These two studies reveal two distinct functions of CB: isoform-specific regulation of gephyrin clustering to promote GABAergic synaptic plasticity and regulation of Cdc42 activity for dendritic maturation, in cooperation with gephyrin and synaptic localization.

I. GENERAL INTRODUCTION

Collybistin (CB) is a neuron-specific guanine nucleotide exchange factor (GEF), highly conserved in vertebrates. It plays a key role in the regulation and plasticity of inhibitory neurotransmission mediated by γ-aminobutyric acid (GABA). In humans, mutations in *ARHGEF9*, encoding CB, cause severe mental retardation and hyperekplexia (Harvey et al., 2008), most likely by disrupting GABAergic function. The aim of my thesis is to investigate the relevance of the structural heterogeneity of CB, arising from alternative splicing, for GABAergic synapse formation and plasticity *in vitro* and *in vivo*.

1 OVERVIEW OF GABAERGIC TRANSMISSION

1.1 Role of GABAergic transmission

All CNS functions, from perceiving sensory stimuli and executing motor tasks to generating emotions and building memory, depend on neuronal integration of excitatory and inhibitory inputs. Through inhibitory neurotransmission, neuronal excitability can be controlled and the firing rate of neurons modulated to synchronize neuronal circuits. The synchronization is important for the emergence of network oscillations, which underlie higher cognitive functions. If inhibition is too weak, anxiety or seizures can result, on the other hand if inhibition is too strong, sedation occurs, up to loss of consciousness and coma. Fast inhibitory neurotransmission in the brain is mediated mainly by the amino acid GABA, whereas glycine primarily mediates fast inhibition in the brain stem and spinal cord. GABA is synthesized by decarboxylation of glutamate by glutamic acid decarboxylase (GAD) and is enriched in presynaptic vesicles by vesicular GABA transporter (vGAT) (McIntire et al., 1997). Upon depolarization of the GABAergic presynaptic terminal by an action potential, intracellular Ca^{2+} concentration raises leading to exocytosis of synaptic vesicles and GABA release in the synaptic cleft, where it binds to GABA receptors. GABA is taken up again by the GABAergic neurons or by glia cells, where GABA is degraded to succinic semialdehyde, a component of the Krebs cycle, by GABA transaminases (GABAT). GABA can activate two receptor types mediating different responses. Fast inhibitory transmission is generated by activating GABA

receptor type A ($GABA_AR$), a ionotropic chloride channel, and slow transmission upon binding to GABA receptor type B ($GABA_BR$), a G-protein coupled receptor, leading to inhibition of adenylate cyclase and voltage-gated Ca^{2+} channels and activation of inward-rectifying K^+ channels. $GABA_AR$ are pentameric, Cys-loop ligand gated ion channels encoded by a family of 19 genes (α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ and ρ1–ρ3). Differential subunit assembly allows the formation of multiple $GABA_AR$ subtypes with specific function and pharmacological properties (Olsen and Sieghart, 2008). $GABA_AR$ mediate two distinct forms of fast inhibition, phasic and tonic. Receptors located at postsynaptic sites produce, upon presynaptic quantal release of GABA, a fast, high amplitude phasic current, also known as inhibitory postsynaptic potential (IPSP). Through the proximity of the receptor to the transmitter release site within the synapse, the kinetics of the IPSP is tightly controlled. Phasic inhibition plays a key role in synchronizing neuronal activity. Tonic inhibition, on the other hand, is mediated by extrasynaptic receptors generating low amplitude currents evoked by ambient GABA spilling over from the synaptic cleft (Farrant and Nusser, 2005). Extrasynaptic $GABA_AR$ are involved in the regulation of neuronal excitability and plasticity (Brickley and Mody, 2012).

Besides being the main synaptic inhibitory neurotransmitter in adult CNS, GABA also plays a role in brain development. Before formation of synaptic contacts, GABA acts as an autocrine and paracrine signaling mediator involved in all major steps of neuronal differentiation (Jelitai and Madarasz, 2005). During fetal and early postnatal life, GABA transmission is excitatory, the opening of the Cl^- channel results in an efflux of Cl^-, depolarizing the cell (Chen et al., 1996). It was shown that depolarizing GABA regulates neuronal proliferation (Obrietan et al., 2002), survival (Obata, 1997), and migration (Behar et al., 2000). GABA depends on chloride-cation co-transporter to establish the Cl^- gradient to act depolarizing. The expression level of K^+-Cl^- cotransporter 2 (KCC2, exporting Cl^-) which counteract Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1, importing Cl^-) is essential, as it is up-regulated during development thus rendering the GABA hyperpolarizing by changing intracellular Cl^- concentrations (Kaila et al., 2014).

1.2 Receptor heterogeneity through differential subunit assembly

Most $GABA_AR$ are composed of two α, two β and one γ/δ/ε subunit. Differential assembly of subunit variants defines $GABA_AR$ subtypes, with specific localization, kinetics properties and pharmacological profile. The GABA binding site lies between the interface of α and β subunits, and its affinity changes with the composition of the receptor. Extrasynaptic receptors typically are composed of α4, α5 or α6 subunits, along with a β and the δ or γ2 subunit, and mediate tonic inhibition through ambient GABA at μM level (Saxena and Macdonald, 1996), whereas synaptic receptors mediating phasic inhibition comprise mainly α1, α2 and α3 subunits, along with a β and the γ2 subunit and need GABA concentrations in the mM range (Perrais and Ropert, 1999). Among these receptors, the α subunit determines their decay kinetic, with α1-$GABA_AR$ being fastest. In addition, the α subunit is important for assembly and surface expression of the receptor, since β and γ subunits alone do not form functional receptor when expressed *in vitro* (Connolly et al., 1996). The relevance of $GABA_AR$ subunit composition was studied by targeted gene deletion with a focus on the function and localization of the receptor. Surprisingly, the different subunit specific gene-deletion showed different levels of compensation by other subunits but no replacement. For example, deletion of the *GABARA1* gene did not cause major behavioral changes, but the network compensated the loss of α1 subunit with an up-regulation of α2 and α3 subunit in other circuits (Kralic et al., 2006). The deletion of the α2 subunits causes some receptor rearrangements in CA1 pyramidal cells, but no replacement by the α1 subunit (Panzanelli et al., 2011). Since the receptor subunits are not merely substituted by other subunits upon deletion, it is impossible to attribute a global function to a specific subunit. This is especially true for subunits like the α5, which are found mostly in extrasynaptic receptors together with β2/3 and γ2 but also at synaptic sites (Serwanski et al., 2006). The γ2 subunit is essential for postsynaptic localization of $GABA_AR$ and its deletion causes postnatal lethality (Essrich et al., 1998). But again, some native $GABA_AR$ receptors containing the γ2 subunit are also located extrasynaptically.

The distribution of the subunits was characterized by non-radioactive *in situ* hybridization and immunohistochemistry, and verified by gene deletion (Fritschy and Panzanelli, 2014). In the adult brain, the γ2 subunit is ubiquitously expressed, as

it is associated with the majority of $GABA_AR$, other than the γ1 subunit, which is restricted to the hypothalamus, amygdala, parts of the basal ganglia and the inferior olivary nucleus. The extrasynaptic counterpart of γ2, the δ subunits, is co-localized with either the α4 subunit in the forebrain or the α6 subunit in the cerebellum. The six α subunits have distinct expression patterns. Whereas the α1 subunit is expressed abundantly throughout the brain, the α2 subunit shows a complementary distribution pattern to the α1 subunit, being highly expressed where α1 subunit expression is low and vice versa. Both subunits are expressed equally only in some brain regions, like the outer layers of cerebral cortex. The α3 subunit shows a similar distribution to the α2, although at a lower level and is also expressed in the brain stem. The α5 subunit is abundant in some restricted areas, namely the olfactory bulb, hippocampus and spinal trigeminal nucleus. β2 and β3 subunit expression overlaps mostly with α1 or α2 subunits, β1 is expressed only at low levels (Fritschy et al., 1994).

In the developing brain, a change in the predominant receptor composition takes place around the period of synaptogenesis. Especially the α5 subunit, thought to mediate tonic inhibition, is highly expressed throughout the brain before synaptogenesis and decreases gradually afterwards (Paysan et al., 1997). Another developmental switch takes place during the first week after birth, when the α2 subunit, which is highly expressed at P0, is subsequently replaced by the α1 subunit (Fritschy et al., 1994). Such replacement results in alteration of GABAergic transmission (Hollrigel and Soltesz, 1997), but the significance of such subunit replacement during development for brain circuit function is still unclear.

$GABA_ARs$ are established phospho-proteins; especially on the β and γ2 subunits multiple phosphorylation sites have been identified. Phosphorylation of $GABA_AR$ regulates important facets of GABAergic synaptic transmission, including receptor trafficking and lateral diffusion, protein-protein interactions, and gating properties. Furthermore, endocytosis of $GABA_AR$ is also dependent on phosphorylation. Several protein kinases located at the postsynaptic density can phosphorylate $GABA_AR$ at the same site (Luscher et al., 2011). Interestingly, phosphorylation of a given residue can lead to different effects, as shown for protein kinase A (PKA), which depresses GABA-activated currents in HEK-293 cells when phosphorylating the β1 subunit,

whereas in β3 subunit-expressing cells the currents get potentiated (McDonald et al., 1998).

Through ubiquitination and subsequent degradation, the $GABA_AR$ availability is regulated on one hand already in the ER before the receptor is trafficked to the membrane (Bedford et al., 2001) and on the other hand after endocytosis, affecting the recycling of the receptor (Kittler et al., 2004).

Thus, posttranslational modification is an additional mechanism to regulate the number and localization of the $GABA_AR$ in a subunit-specific manner, contributing to heterogeneity in the regulation of GABAergic transmission.

1.3 Interneurons mediate most of the GABAergic inhibition

The broad diversity of $GABA_AR$ subtypes, with the possibility to regulate the surface expression in a spatial and temporal manner, is needed for the neurons to respond to the different inputs in a precise way, for effective neuronal computation and network synchronization. Not only the postsynaptic site is specialized, but also the presynaptic terminals arise from a variety of highly differentiated neurons, termed interneurons, which innervate different subcellular domains on their postsynaptic targets.

Most interneurons are GABAergic innervating only nearby neurons, which distinguishes them from projecting neurons (principal cells) connected to distant brain regions. Interneurons are important for the formation of functional assemblies of small subsets of principals cells, controlling their firing rate and spike timing, thereby synchronizing their activity (Klausberger, 2009). In the hippocampus, interneurons innervating pyramidal cells in the CA1 region are among the best characterized. They are classified according to their firing properties, molecular expression profiles, such as parvalbumin (PV) or cholecystokinin (CCK), and the innervation pattern of distinct subcellular domains of pyramidal cells (Klausberger and Somogyi, 2008). Axo-axonic cells innervate pyramidal cells exclusively on the axon initial segment; basket cells, the most prominent interneurons in the hippocampus, innervate mainly the perisomatic region of pyramidal cells. These two classes are especially interesting, since they have the potential to control the output of pyramidal cells. The majority of interneurons in CA1, however, innervate the

dendrites of pyramidal cells and contribute to the integration of information and synaptic plasticity. These include, for example, bistratified cells, innervating basal and oblique dendrites and O-LM cells (having their name from the location of the cell body in stratum oriens (O) and innervating apical dendrites of principal cells in the stratum lacunosum-moleculare (LM)), aligned with entorhinal cortex input.

Basket cells are a good example to illustrate how the specialization of pre-and post-synaptic elements contribute to generation of distinct outputs of the principal cell. PV-containing basket cells are fast-spiking interneurons interconnected by GABAergic synapses and GAP junctions and receiving extensive glutamatergic input from Schaffer collaterals; they are involved in generating high-frequency (gamma range) network oscillation. CCK-positive basket cells are regular spiking neurons receiving moderate inputs from Schaffer collaterals, as well as serotonergic input from the median raphe; they are involved in fine-tuning of network activity and behavioral states (Freund and Katona, 2007). Synapses of PV-positive cells contain α1 $GABA_AR$ (Klausberger et al., 2002), while CCK-positive basket cells form synapse containing α2 $GABA_AR$ (Nyiri et al., 2001). This difference shows the specificity of $GABA_AR$ subtypes to presynaptic influences.

1.4 Pharmacological relevance of $GABA_AR$

$GABA_AR$ are the specific site of action of benzodiazepines (BZD), which act as allosteric modulators of the GABA binding site. The effects of BZD are sedative, hypnotic, anxiolytic, anticonvulsant and muscle relaxant; in high doses even amnesic. The effects of BZD can be blocked by flumazenil, a competitive antagonist at the BZD binding site. In pentameric $GABA_AR$, GABA binds at the interface between the α and β subunit, inducing a conformational change opening the Cl^--channel. The binding of GABA can be positively or negatively modulated by ligands of the BZD site, located at the interface of the α and γ subunits. Binding of BZD agonists increases the opening frequency of the Cl^--channel, thus potentiating GABA. The study of this binding site revealed important information about the function of these receptors leading to a classification of diazepam-sensitive receptors containing the α1, α2, α3 and a5 subunit and diazepam-insensitive receptors, containing α4 and α6 subunits. Mutating a conserved histidine residue (H101 for α1 and α2 and H105 for α5) in the drug binding pocket to an arginine rendered the receptor insensitive to diazepam

without altering its GABA sensitivity and gating properties (Rudolph et al., 1999). *In vivo*, the analysis of knockin mice carrying diazepam-insensitive $GABA_AR$ subtypes revealed that each α subunit variant mediates distinct actions of diazepam. The α1-containing receptors mediate sedation and anterograde amnesia, while α2-containing receptors mediate anxiolysis (Rudolph et al., 2001). Last, mice carrying the H105R point mutation in the α5 subunit exhibit improved memory function in specific hippocampal dependent tasks (Crestani et al., 2002). Furthermore, a recent study showed anti-hyperalgesic effects mediated by the α2 subunit of $GABA_AR$ located in the spinal cord. This effect was achieved by systemic administration of a non-sedative BZD site agonist HZ166 and had its origin exclusively in the spinal cord (Paul et al., 2014).

Neurosteroids, like allopregnanolone and tetrahydro-deoxycorticosterone (Belelli and Lambert, 2005), are synthesized in the brain during stress, pregnancy, and alcohol intoxication. In low concentrations (nano-molar range), they bind at a transmembrane site on the α subunits, potentiating the GABA response. In a sub-micro- to micro-molar range, neurosteroids activate directly the receptor (Hosie et al., 2006). Perturbations in neurosteroid levels are associated with psychiatric disorders, including major depression and schizophrenia. As they can act pre-synaptically, modulating vesicular release, or post-synaptically, and even extra-synaptically, neurosteroids offer multiple mechanisms for modulating neuronal excitation in response to stress (Herd et al., 2007). Another endogenous modulator of $GABA_AR$ is the family of endozepines, which bind to the same sites as BZD and affect the receptors in the opposite way (Christian et al., 2013). Exogenous compounds acting on $GABA_AR$ are ethanol and general anesthetics, with the difference that they have also other targets in the brain (Grasshoff et al., 2007).

GABA binding site agonists like muscimol, and antagonists like bicucculine or gabazine, are known. There is no clinical use for these agents, but they are important for experimental studies of $GABA_AR$ functions. Noteworthy is that phasic inhibition is gabazine-sensitive and tonic inhibition is relatively gabazine-insensitive (Yeung, et al., 2003).

BZD site-ligands are extensively used for treating anxiety and sleeping disorders, as well as muscle spasms, but their long-term use is limited by onset of tolerance and dependence. Even upon a single BZD administration, surface expression of $GABA_AR$ is changed. In addition as most of the BZDs are not $GABA_AR$ subtype specific, other side effects, especially sedation, impaired motor coordination, and anterograde amnesia, are also issues. BZD replaced barbiturates to treat sleeping disorders since BZD are much safer as they only act as allosteric modulators of $GABA_AR$ and they do not induce drug metabolism. However, the design of new derivatives acting only on specific $GABA_AR$ subtypes would be a major advance to separate anxiolysis from sedation and/or preventing dependence.

1.5 Pathologies of disturbed GABAergic transmission

The heterogeneity of $GABA_ARs$ subtypes underlies their circuit-specific functions, which are also mirrored in the wide spectrum of neurological and psychiatric diseases involving altered GABAergic transmission. In patients with idiopathic epilepsies, for example, mutations of several $GABA_AR$ subunits have been identified. The form of epilepsy can vary in severity and depends on the location of the gene mutation. Mutations in the coding sequence for the δ or γ2 subunit affect the kinetics of the receptor by reducing the mean open time (Baulac et al., 2001; Dibbens et al., 2004), or, as seen in the α1 or β3 subunit, reduce protein levels and surface expression (Cossette et al., 2002; Tanaka et al., 2008).

A regional loss of neurons can arise from several causes like trauma, stroke, or a status epilepticus (SE). In pathology, the peri-lesion tissue undergoes plasticity changes to recover functionality, especially tonic inhibition is increased by the recruitment of extrasynaptic $GABA_ARs$, leading to a change of neuronal excitability (Zhang et al., 2007; Cramer, 2008). Interestingly, administration of BZD inverse agonist in the peri-infarct area after stroke down-regulated tonic inhibition, enhancing functional recovery (Clarkson et al., 2010).

Keeping the balance between excitation and inhibition is not only important in plasticity after brain lesion to remap the neuronal circuits, but also in plasticity during development. Even minor changes in the E/I balance during these critical

windows can result in altered information processing leading to an impairment of sensorimotor and cognitive development (Hensch, 2005).

Different mechanisms that alter GABAergic transmission have been linked to brain diseases. Anxiety and depressive disorders can be modelled in mice heterozygous for the γ2 subunit gene. Since it was thought that the origin for these diseases lies in early childhood, the γ2 subunit was silenced at two early time-points of development. Altering GABAergic transmission around P13 resulted in increased anxiety-like behavior and around P28 caused increased immobility behavior in the forced-swim test, a readout for depression. These findings demonstrate that the sensitivity to GABA during development controls separate critical periods, and if altered can lead to different mood disorders (Shen et al., 2012).

In neuropathology such as schizophrenia, altered prenatal development is suggested to lead to dysfunction of specific interneurons in prefrontal cortex (Volk and Lewis, 2014). Moreover, studies of post-mortem brains from schizophrenic patients revealed reduced Reelin levels, affecting neuronal migration prenatally and synaptic plasticity postnatally (Tueting et al., 2006) and causing a shift towards increased expression of extrasynaptic $GABA_AR$ (Ishikawa et al., 2004). These findings illustrate the complexity of the pathophysiological mechanisms underlying these diseases and how disturbances affecting the GABAergic system at different time-points during development and location in the brain can lead to psychotic disorders. The treatment with BZD might be beneficial to normalize GABAergic transmission, but it would need to be specific for extrasynaptic $GABA_AR$ to reduce side effects, such as sedation or tolerance (Guidotti et al., 2005).

These few examples show the importance of GABAergic transmission for brain development and function, and the many facets of psychiatric disorders, when it is altered. A better understanding of the mechanisms underlying $GABA_AR$ expression and targeting might help to develop novel therapeutics to neurological and psychiatric diseases.

2 MOLECULAR ORGANIZATION OF THE GABAERGIC POSTSYNAPTIC DENSITY

The postsynaptic density (PSD) is an electron dense region in the postsynaptic membrane facing the active zone of the presynaptic terminal. The PSD contains a protein scaffold anchoring the receptors close to the neurotransmitter release site. This scaffold also organizes a signaling complex by concentrating different effector proteins, kinases, and phosphatases close to the receptor, allowing a fast signal transduction to local protein activation or nuclear protein transcription (Figure I). The composition of the PSD varies between brain regions and neurotransmitter systems and is best characterized for glutamatergic synapses, which are for the most part located on spines. In the next sections, the PSD protein of GABAergic synapses are introduced, which are important for this thesis.

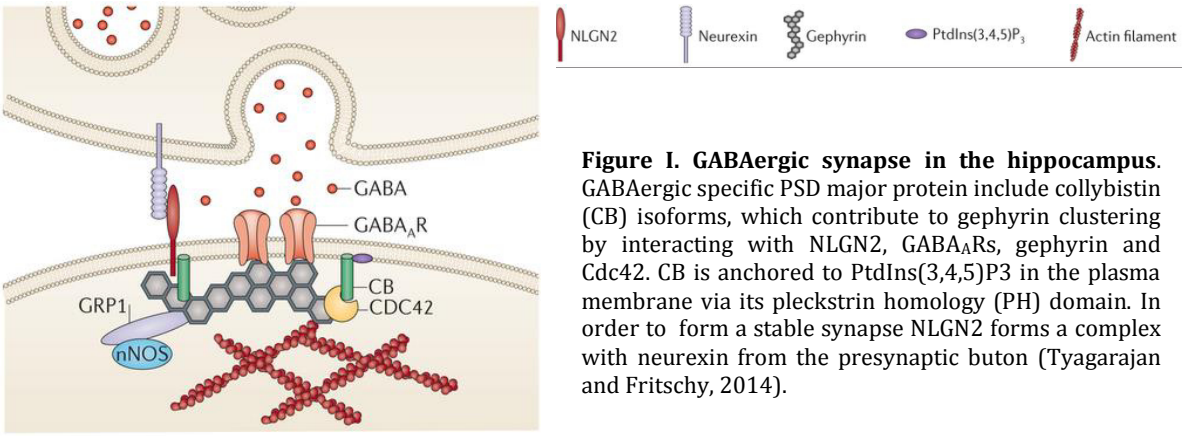

Figure I. GABAergic synapse in the hippocampus. GABAergic specific PSD major protein include collybistin (CB) isoforms, which contribute to gephyrin clustering by interacting with NLGN2, $GABA_ARs$, gephyrin and Cdc42. CB is anchored to PtdIns(3,4,5)P3 in the plasma membrane via its pleckstrin homology (PH) domain. In order to form a stable synapse NLGN2 forms a complex with neurexin from the presynaptic buton (Tyagarajan and Fritschy, 2014).

2.1 Gephyrin

One of the essential molecules at the PSD of GABAergic synapses is gephyrin, which selectively aggregates postsynaptically, forming a dynamic scaffold anchoring receptors and other proteins at the PSD. Gephyrin is a highly conserved multi-functional protein, required throughout the living kingdom for catalyzing molybdenum cofactor (MoCo) biosynthesis. Gephyrin was first identified as a component of GlyR in neurons, and shown later to be a cytoplasmic protein, bridging the receptor to the cytoskeleton (Pfeiffer et al., 1982). Subsequently, gephyrin was found to be also associated with GABAergic PSD (Sassoe-Pognetto et al., 2000). Independent studies have identified a direct interaction between gephyrin and the α1-α3 $GABA_AR$ subunits (Tretter et al., 2008; Saiepour et al., 2010), as well as with the β2 and β3 $GABA_AR$ subunits, albeit with lower affinity compared to the GlyR β

subunit (Maric et al., 2011). Down-regulation of gephyrin in cultured neurons and its targeted deletion in mice reduced α2 and α3 $GABA_AR$ subunit-containing postsynaptic clusters (Essrich et al., 1998; Kneussel, 2001), indicating a role of gephyrin in $GABA_AR$ clustering. However, α1 subunit clusters were not lost, indicating a $GABA_AR$ clustering independent of gephyrin (Levi et al., 2004). Through the study of γ2 subunit knock out mice, where functional $GABA_AR$ could still be formed but gephyrin cluster were lost at the postsynaptic site, it became evident that gephyrin clustering depends on $GABA_ARs$ (Essrich et al., 1998). These findings were strengthened by the observation in mice lacking the α2 subunit, where the majority of gephyrin clusters are lost in hippocampal pyramidal cells, for example. Interestingly, the frequency of mIPSC was only moderately reduced, indicating a compensation of the lost receptors by α1 subunit containing receptors, independent of gephyrin (Panzanelli et al., 2011).

Structurally, gephyrin is composed of a conserved 20 kDa N'-terminal G- domain and a 43 kDa C'-terminal E- domain linked by an 18-21 kDa unstructured C-domain (Lardi-Studler et al., 2007). The G- and the E-domain are homologous the bacterial MogA and MoeA proteins, carrying out the MoCo synthesis. The C-domain renders the full-length gephyrin flexible but is highly susceptible to proteolytic cleavage (Herweg and Schwarz, 2012). Due to the instable linker, the structure of full-length gephyrin could not be determined by crystallography, but studies from isolated domains showed that the G-domain can form trimers and the E-domain dimers. However, full length gephyrin forms only trimers, as the dimerization of the E-domain is inhibited (Sander et al., 2013). Through conformational changes of the linker region, gephyrin trimers have either a globular or an extended structure and auto-regulatory mechanisms are proposed with regard to the formation of a hexameric lattice that anchors the receptors at the PSD. Since the C-domain is solvent exposed, it is a potential target for protein-protein interactions and post-translational modifications.

Gephyrin post-translational modification has emerged as an important determinant of its postsynaptic scaffolding properties, and thereby of the regulation of GABAergic synaptic transmission and plasticity (Tyagarajan and Fritschy, 2014). Phosphorylation as a post-translational modification of gephyrin has been identified

already for some time (Langosch et al., 1992), but it was only recently demonstrated that the residues Ser28 and Ser270 play an important role for the regulation of GABAergic synapse formation and size.

The expression of phospho-deficient gephyrin mutants at Ser268 in cultured neurons led to an increase of gephyrin cluster size and we could demonstrate that Extracellular Signal-regulated Kinase (ERK) mediates phosphorylation at Ser268. The increase of gephyrin cluster size was also mirrored by changes of amplitude and frequency in miniature GABAergic postsynaptic currents (Tyagarajan et al., 2013). The phosphorylation status at Ser268 has an influence on glycogen synthase kinase 3β (GSK3β)-mediated phosphorylation of gephyrin on Ser270, which negatively regulates gephyrin cluster density (Tyagarajan et al., 2011b). Through phosphorylation of Ser270 a conformational change takes place and makes gephyrin susceptible to Ca^{2+} dependent proteolytic cleavage by calpain1 (Tyagarajan et al., 2011b). These findings revealed an activity-dependent regulation of gephyrin leading to changes in GABAergic postsynaptic currents.

Recently, we have also identified SUMOylation and acetylation as two new posttranslational modifications of gephyrin. The characterization of specific resides that are SUMOylated and acetylated demonstrated the importance of these two modifications, along with phosphorylation, for gephyrin scaffold formation at GABAergic synapses (Tyagarajan et al., 2011b; Tyagarajan et al., 2013).

A further posttranslational modification of gephyrin is palmitoylation, which is regulated by $GABA_AR$ activity and governs membrane anchoring of gephyrin and thereby strengthens GABAergic transmission (Dejanovic et al., 2014). The regulation of gephyrin through posttranslational modification and the effect it has on the synaptic strength changed the picture of gephyrin from a static protein anchoring receptors to the cytoskeleton to a dynamically regulated scaffolding protein mediating activity-dependent plasticity.

2.2 Interactors of gephyrin

Besides $GABA_AR$, gephyrin interacts with several other proteins at the PSD, including cytoskeletal, signaling, and trans-membrane proteins (Tyagarajan and Fritschy, 2014). Only the ones relevant for this thesis will be discussed below.

Neuroligin 2

In order to form a functional synapse, the pre- and postsynaptic components need to be linked together. Neuroligins are synaptic adhesion molecules with one transmembrane domain, spanning the synaptic cleft to interact with presynaptically located neurexins (Lisé and El-Husseini, 2006). Four different genes encode neuroligins (Bolliger et al., 2001), but only neuroligin2 (NLGN2) is exclusively located at GABAergic synapses (Varoqueaux et al., 2004). Both neuroligins and neurexins are capable to trigger synapse formation *in vitro* (Scheiffele et al., 2000; Graf et al., 2004) and the interaction between these two proteins is regulated through Ca^{2+} (Araç et al., 2007). Neuroligin2 (NLGN2) can directly interact with gephyrin and CB (Poulopoulos et al., 2009), which is necessary for postsynaptic localization of gephyrin. *In vivo* studies of NLGN2-KO mice showed a reduction of GABAergic transmission in the hippocampus via a loss of gephyrin and perisomatic GABAergic synapses in principal neurons (Jedlicka et al., 2011). Deletion of either neurexin or neuroligin *in vivo* showed the important role of these proteins for proper synaptic function, as synaptic transmission was affected, but more research is needed to determine the significance of the complex between neuroligin and neurexin for synapse formation, as the number of synapses was not significantly altered (Südhof, 2008).

Collybistin

Collybistin (CB) is a guanosine nucleotide exchange factor (GEF) belonging to the family of dbl RhoGEFs. It was first identified in a yeast-two hybrid screen of binding partners of gephyrin, and shown to translocate gephyrin from intracellular aggregates to the cell surface in a recombinant expression system (Kins et al., 2000). CB is encoded by a single gene, *ArhGEF9*. In rat, there are six isoforms known so far, generated by alternative splicing. Their main difference between CB1-CB3 is the length and amino acid sequence of the C'-terminus. In addition each of these CB isoforms exists with or without an N-terminal SH3 domain (SH3+/SH3-) (Figure

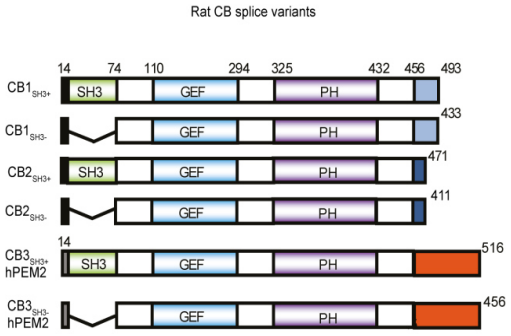

Figure II Protein domains of rat CB splice isoforms. CB3 is identical with the human analogue hPEM2

II). *ArhGEF9* is present in all vertebrates and specific for the CNS; CB3 is the homologue to the human hPEM2. The disruption of the gene in humans leads to severe cognitive deficits, epileptic seizures, and hyperekplexia. Interestingly, targeted deletion of CB in mice does not lead to a severe or lethal phenotype. Behaviorally, the CB-KO mice show an anxiety like phenotype, but no deficits in motor function (Papadopoulos et al., 2007).

Structure and Domains

Dbl-family RhoGEFs are characterized by a conserved tandem domain: the dbl homolog domain (DH) executes the catalytic exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) on small RhoGTPases (see Box 1), and the pleckstrin homolog domain (PH) can interact with phosphatidylinositol-(3,4,5)-triphosphate (PIP3) and thus anchor CB to the membrane. In addition, they harbor an N-terminal src-homolog 3 domain (SH3). CB is a RhoGEF specific for Cdc42 and TC10, which also belongs to the Cdc42 family (Reddy-Alla et al., 2010; Mayer et al., 2013). Membrane-anchoring of CB through the PH domain is essential for its regulation of gephyrin trafficking and postsynaptic clustering, as shown by overexpression of $CB2_{SH3-}$ lacking the PH domain in cultured hippocampal neurons (Harvey et al., 2004). However, overexpressing a constitutively active Cdc42 mutant in these cells restores gephyrin clustering (Tyagarajan et al., 2011a), indicating that Cdc42 can substitute CB at GABAergic synapses. Regarding the facilitation of gephyrin clustering when CB is overexpressed in neuronal cultures, the SH3 domain has only minor effects. However, the SH3 domain has important features; it harbors a binding site for NLGN2 and $GABA_AR$ α2 and α3 subunits. Furthermore, the SH3 domain modulates the conformation of CB through intramolecular interactions. It was suggested that the SH3 domain can auto-inhibit CB binding to plasma phosphoinositides by masking the DH/PH tandem domain. Upon binding to NLGN2, the conformation opens and phosphoinosisitide binding can take place, stabilizing CB at the synapse (Soykan et al., 2014). The C'-terminus is unique to each CB isoform and harbors a coil-coiled domain, which is thought to play a role in protein-protein interaction. Through differences in positively charged residues, the ability to interact with the membrane could be altered between the isoforms, as suggested by Xiang et al. (Xiang et al., 2006). It is one of the main aims of this work to investigate

the functional differences between CB isoforms for GABAergic synapse formation and plasticity.

Box 1 RhoGTPases and RhoGEFs

Rho-family GTPases contain at least 20 different members, and RhoA, Rac1 and Cdc42 are the best studied. At the beginning RhoGTPases were thought to play only a role in cytoskeleton rearrangement, enabling cell migration and polarity. It was shown that RhoA and Rac1 can trigger actin bundle reorganization leading to the formation of lamellipodia and stress fibers, whereas activated Cdc42 induces filopodia (Nobes, 1999). Later, it was discovered that they also play a role in intracellular trafficking, cell growth, and synapse remodeling. By activating multiple signaling pathways they operate as a regulatory switch impacting a wide range of cellular functions. Because of their ability to act on many targets, RhoGTPases need to be regulated in a spatially and temporally controlled manner. RhoGTPases act as a molecular switch cycling between inactive GDP-bound and active GTP-bound states (Figure III). To be activated they need RhoGEFs, which catalyze the exchange of GDP for GTP. There is a broad range of RhoGEFs expressed in the brain, specific to one or more small RhoGTPases. Each RhoGTPase can be activated by multiple GEFs. The role of the GEFs is not simply to activate the RhoGTPase, but also to form complexes and thereby translocate the RhoGTPases to the right place and in the proximity of other effector proteins (Sinha and Yang, 2008).

Besides the conserved DH and PH domain, RhoGEFs can harbor other domains, which allow them to exert a specific role, either in a specific subcellular location or through protein-protein interactions. Sometimes, it is sufficient to change splicing of a Rho-GEF to change its role in the cell. Although many of the RhoGEF proteins are expressed as alternative splice isoforms, the exact function and localization of these isoforms are only known for a small subset of RhoGEFs. A well-studied example is Kalirin, a multi-domain dual RhoGEF (Johnson et al., 2000; McPherson et al., 2002; Rabiner et al., 2005). Alternative splicing of Kalirin affects whole domains, for example removing one DH/PH tandem domain, and the isoforms are differently expressed during development. While Kalirin12 is located uniquely in the soma, Kalirin9 is targeted to dendritic spines, increasing the size and number of spines (McPherson et al., 2002).

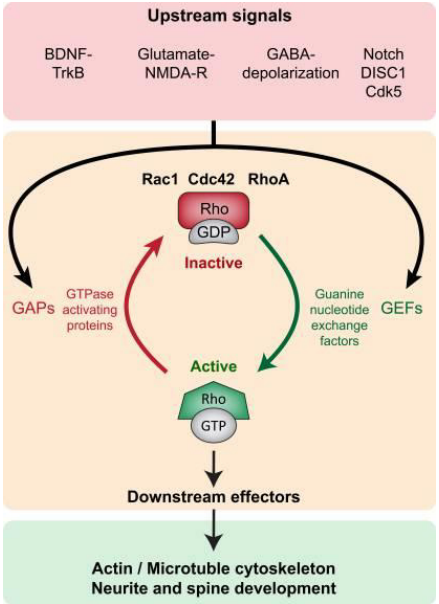

Figure III Small Rho GTPase signaling in neurite maturation. Upstream signals modulate GEFs and GAPs thereby regulating the activity of small Rho GTPases Rac1, Cdc42, and RhoA. GEFs promote activation (GTP-bound conformation) and GAPs promote inactivation (GDP-bound conformation). In the active state, small Rho GTPases bind to several downstream effectors exerting influence on local actin and microtubule networks (Vadodaria and Jessberger, 2013).

Synaptic function

The importance of CB at GABAergic synapses became evident through the knockout of CB in mice. The loss of CB led to a region-specific loss of gephyrin clusters together with $GABA_AR$ clusters in the hippocampus and basolateral amygdala. The decrease in $GABA_AR$ reduced dendritic GABAergic inhibition and increased network excitability, which impaired long-term potentiation (Papadopoulos et al., 2007; Jedlicka et al., 2009).

Conversely, in cultured neurons, it was shown that CB overexpression increases gephyrin cluster number and density in an isoform-specific manner, which is accompanied by increased GABA-mediated currents (Chiou et al., 2011; Tyagarajan et al., 2011a). Furthermore the deletion of the PH domain in CB keeps gephyrin in long intracellular aggregates and the disruption of the DH domain reduces gephyrin cluster density, demonstrating the need of CB to translocate gephyrin to the post synapse and stabilization at the synapse. The importance of CB and gephyrin interaction was also shown by a down-regulation of CB in cultured neurons, which led to reduced phosphorylation of gephyrin at Ser270 mediated by Cyclin dependent kinase 5 (Cdk5) (Kuhse et al., 2012). Since the interaction between CB and gephyrin differs between isoforms and has different effects (Chiou et al., 2011; Tyagarajan et al., 2011a), it is of great relevance to further study the role and function of CB splice isoforms.

The role of Cdc42 activation by CB is currently unclear; however, the biochemical evidence supports direct interaction between CB and Cdc42. TC10 was recently identified to interact with CB and shown to play an essential role in CB activation (Mayer et al., 2013). TC10 promotes CB recruitment to the plasma membranes via a currently unknown mechanism. Hence, the present thesis focused only on Cdc42 interaction with CB and examined the biochemical basis for CB function. *In vitro* binding studies showed the formation of a ternary complex of CB2 gephyrin and Cdc42, which is favored when CB2 is lacking the SH3 domain. Furthermore, in cultured neurons Cdc42 co-transfected together with gephyrin and CB2 had an effect on gephyrin cluster size and synaptic localization depended on the activation state of Cdc42 (Tyagarajan et al., 2011a). Since both constitutively active Cdc42 and dominant negative Cdc42 had an effect on gephyrin clustering, the role of CB acting

as a GEF at the GABAergic synapse is questioned. Another study, by using point-mutations in the GEF domain of CB, showed that the activation of Cdc42 is not required for gephyrin clustering at the post synaptic site (Reddy-Alla et al., 2010). These findings are in line with a systematic screen of the Dbl family to characterize their selective activation of RhoGTPases, which revealed that CB has only low catalytic activity toward Cdc42 or TC10 (Jaiswal et al., 2013). Thus, it is not surprising that it is still debated whether the GEF activity of CB is needed for Cdc42 activation at GABAergic synapses, raising the question about the functional role of CB interaction with Cdc42, and whether there are differences between CB splice isoforms as these experiments were only performed with CB2.

3 THE ROLE OF GABA IN NEURONAL MATURATION

It has been recognized long ago that GABAergic transmission operates early during fetal development to regulate neuronal proliferation, differentiation, and functional maturation prior to the establishment of excitatory synaptic connections. The diversity and plasticity of GABAergic synapses provides a vast array of mechanisms underlying the neurodevelopmental effects of GABA. However, there is little evidence, so far, how regulators of gephyrin, notably activators of the cytoskeleton, contribute to these effects.

3.1 Neuronal ontogeny

During early development, GABA influences major steps of neuronal differentiation. Proliferation of progenitor cells is regulated by the inhibition of DNA synthesis upon depolarization mediated by GABA release (LoTurco et al., 1995). Interestingly, GABA promotes proliferation in the ventricular zone (VZ), whereas it decreases proliferation in the subventricular zone (SVZ) (Haydar et al., 2000). GABA also acts in two ways on the migration of the neurons, it promotes migration via $GABA_BR$ and G-protein activation and arrests migration via $GABA_AR$-mediated depolarization (Behar et al., 1998). Dendrite outgrowth and branching are decreased upon antagonizing GABA by bicuculline in cultured embryonic hippocampal neurons. Agonizing GABA by muscimol did not cause any effect (Barbin et al., 1993). Thus, GABA has a major effect on the development of cortical regions. Sensory deprivation in early development induces lasting structural and functional changes in the cortex, but only during critical period windows. Their opening time can be modulated by

reducing or enhancing GABAergic transmission, demonstrating the role of GABA for integration and network formation during development (Hensch, 1998). These findings underscore the role of GABA in major steps of neuronal differentiation and maturation, but they show also the need to further investigate the $GABA_AR$ subtypes responsible for mediating these effects and how they are regulated.

3.2 Adult neurogenesis

When studying the GABAergic synapse and the function of the multiple postsynaptic proteins, it is essential to investigate an intact system *in vivo* in order to characterize the mechanisms underlying the effects of GABA on neuronal maturation. For this reason, we used in this study adult neurogenesis as a model. Without confounding factors of development, neurons can be studied during a critical time-period, when the cell differentiates and forms synaptic contacts. Since CB was shown to play a role in synapse formation and interact with Cdc42, an actin skeleton remodeling protein, it is potentially of great interest to investigate the role of CB during adult neurogenesis.

In the adult rodent brain, there are two distinct neurogenic niches, the subventricular zone (SVZ) and the subgranular zone (SGZ), which contain stem cells and precursor cells giving rise to new neurons throughout life. While neuronal progenitor cells (NPC) from the SVZ migrate along the rostral migratory stream to the olfactory bulb, NPC from the SGZ migrate only a short distance to integrate into the granule cell layer (GCL) of the dentate gyrus. These newborn neurons differentiate and integrate into the existing circuits and thus play an important role in memory and consolidation function. Disrupted adult neurogenesis is linked to diseases like depression and schizophrenia (Schoenfeld and Cameron, 2014). During the maturation of newborn neurons, they undergo several distinct steps, which are all regulated and influenced by GABA and GABAergic transmission. Proliferation of NPCs is negatively controlled by the γ2 and α4 $GABA_AR$ subunits, as knock down of these subunits in mice increased proliferation (Duveau et al., 2011; Song et al., 2012). Next, the NPCs need to migrate into the GCL and GABA also plays a role in this process, which is not yet fully understood, as many regulating factors other than GABA are thought to be involved. Ablating α2-$GABA_AR$ promotes cell migration, whereas ablating α4-$GABA_AR$ disables the entry into the GCL (Duveau et al., 2011).

The maturation of newborn neurons takes around 6 weeks. During the first week, the neurons have not yet formed functional synapses and respond to ambient GABA, meditating the first steps of integration (Dieni et al., 2012). During this time, GABA has a depolarizing action on these cells, due to the high expression of NKCC1 which imports Cl^- into the cell (Cherubini et al., 1991). Under such conditions, opening of the $GABA_AR$ leads to an efflux of Cl^-, thus depolarizing the cell. Later on, a switch from NKCC1 to KCC2, which exports Cl^-, takes place turning GABA hyperpolarizing (Ge et al., 2006). After two weeks, functional GABAergic synapses are formed and the immature granule cells extend spineless dendrites into the molecular layer, where they make synapses with perforant path fibers (Zhao et al., 2006; Niu et al., 2014). A critical time window starts now, as glutamatergic synapses are formed and become functional. In this period, the survival of newborn neurons can be increased by enriching the environment, leading to increased spine development and thus showing the importance for integration of newborn neurons into the existing circuit (Ge et al., 2008). At around four weeks of age, the neurons have the highest spine density, which is followed by a period of pruning, till the neurons are considered adult at the age of around six weeks (Toni and Sultan, 2011). Ablating different proteins can perturb each of these steps from proliferation to the mature neurons and thus gives answers to the role of these proteins and to possible rescue mechanisms of the neurons. The knock down of α2 subunit, for example, resulted in the opposite phenotype than the knock down of α4, pointing to the balance between phasic and tonic inhibition (Duveau et al., 2011). Expression of dominant negative forms of Cdc42 and Rac1 showed delayed maturation of new born neurons, Cdc42 was involved in early and late development, whereas Rac1 only in the late stages (Vadodaria et al., 2013). Adult neurogenesis as a model can help to gain a better understanding in the complex interaction of synaptic circuits and the role CB might play.

II. AIMS OF THE THESIS

CB is important for the formation and maintenance of GABAergic synapses. The current view posits that CB is activated by a conformational change upon SH3 domain-mediated binding to NLGN2; thereby, CB stabilizes gephyrin at the GABAergic PSD, thus enabling scaffold formation and clustering of $GABA_A$Rs. Through the generation of CB-KO mice, a region- and GABAergic synapse-specific role for CB was demonstrated, also translating in anxiety-like behavioral changes, without motor deficits. Furthermore *in vitro* studies showed that overexpression of CB in primary neuronal cultures leads to an increase in gephyrin cluster size and density, and consequently GABAergic transmission. Crystallographic studies revealed the highly dynamic structure of CB and showed that its structural integrity is essential for CB-mediated clustering of gephyrin at the membrane. The presence of CB isoforms suggests that additional regulatory steps might be at play that are currently not well understood. In addition, it has been shown that the GEF activity of CB is not needed to target gephyrin to the GABAergic synapse, which raises the questions, what is the role of the GEF domain, and which is the function of CB-mediated Cdc42 activation?

In order to address these issues in my PhD thesis, I used an *in vitro* and an *in vivo* approach. By using various recombinant expression systems *in vitro*, I focused on the CB1 and CB2 isoforms for their interaction partners, mRNA levels, protein stability, and effect on postsynaptic gephyrin clustering. These experiments aimed to unravel isoform-specific regulation mechanisms of CB and thus better understand their contribution to GABAergic function and plasticity. In the *in vivo* study, I focused on the relevance of CB overexpression or silencing for the maturation and integration of adult-born GCs. Given the importance for GABAergic signaling for the development of newborn neurons, these experiments allowed studying the role of CB in an intact network and uncovered mechanisms how GABAergic transmission regulates maturation and synaptic plasticity through postsynaptic proteins.

***In vitro* Study:** **Activity-dependent alternative splicing of collybistin alters ubiquitin-dependent protein stability and localization to dynamically modify GABAergic synapses**

Although it is well established that *ArhGEF9* mRNA is spliced in three main isoforms, differing in their C'-terminus, most of the studies focused on the function of the canonical domains of CB for gephyrin clustering and GABAergic synapse formation and ignored its molecular diversity. The importance of $CB1_{SH3-}$ has even been questioned, since it is detectable only in small amounts. In my work, I aimed to fill this gap and investigate the role of the different CB splice variants for GABAergic synapse formation and plasticity. To this end, we used molecular biology and biochemistry to investigate post-transcriptional regulation of CB mRNA splicing and post-translational regulation of CB isoform stability and function. In particular, we worked with purified proteins or overexpressed proteins from HEK-293 cells to determine protein interactions and turn-over. Furthermore, we overexpressed gephyrin, CB splice variants and mutant constructs in cultured hippocampal neurons to analyze the effect of specific mutations on GABAergic synapse formation and morphology.

***In vivo* Study: Collybistin-dependent maturation and synaptic integration of adult born neurons in the hippocampus**

The role of CB in stabilizing gephyrin at the GABAergic synapse does not offer insights into CB as a GEF selectively activating Cdc42 and TC10. It was shown that Cdc42 is important for immature neurons to establish a dendritic tree and integration by modulating the morphology of spines. Furthermore, mice lacking the α2 subunit of $GABA_AR$ show cell autonomous alteration in adult neurogenesis, namely adult-born GCs migrate deeper into the GCL and the dendritic arborization is altered, suggesting a regulatory relationship between cytoskeletal rearrangements and GABAergic transmission. To investigate this relationship and the possible involvement of CB, I have chosen adult neurogenesis as an *in vivo* model to analyze synaptogenesis and neuronal maturation. The aim of this study was to investigate the effect of CB over-expression or silencing on neuronal maturation and synaptogenesis. Furthermore, I investigated whether CB overexpression can rescue in a cell autonomous manner adult-born GCs from the consequences of targeted deletion of the $GABA_AR$ α2 subunit. To address these issues, I used retroviruses expressing eGFP or eGFP-$CB1_{SH3-}$ for overexpression experiments, or panCB shRNA to silence CB expression, in adult-born GCs in the hippocampus and monitoring their morphological development and synaptic integration at specific developmental steps known to be regulated by GABAergic transmission.

III. RESULTS

IN VITRO STUDY: ACTIVITY-DEPENDENT ALTERNATIVE SPLICING OF COLLYBISTIN ALTERS UBIQUITIN-DEPENDENT PROTEIN STABILITY AND LOCALIZATION TO DYNAMICALLY MODIFY GABAERGIC SYNAPSES

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Submitted for Publication

My contribution to this study was the generation of all constructs, protein stability assay, the overexpression and analysis of wild-type hippocampal neurons and the treatment of cortical neurons and mRNA isolation for further qPCR analysis, as well as in the writing process.

ABSTRACT

The function of neuronal circuits depends on a dynamic equilibrium between excitatory and inhibitory transmission. Accumulating evidence shows GABAergic synapses as dynamic structures undergoing activity-dependent plasticity, in part by the modulation of gephyrin scaffold via intra-cellular signaling cascades. Here, with a focus on a GDP/GTP-exchange factor (GEF), collybistin (CB), we show that it functions downstream of synaptic $GABA_A$Rs to recruit gephyrin and promote scaffold formation. We report a highly selective CaMKII/IV-dependent splicing regulation of CB isoforms in response to neuronal activity. In turn, we show that CB protein isoforms are differentially modified by ubiquitin to possess different stabilities with direct influence on gephyrin synaptic scaffolding. By investigating the localization of CB mRNA transcripts, our data shows dendritic transport of CB isoform mRNAs, implicating local protein synthesis as an essential component of GABAergic synaptic plasticity. Our data illustrate that CB splice isoforms provide the molecular diversity to select from a repertoire of interaction partners to shape GABAergic postsynaptic architecture and function.

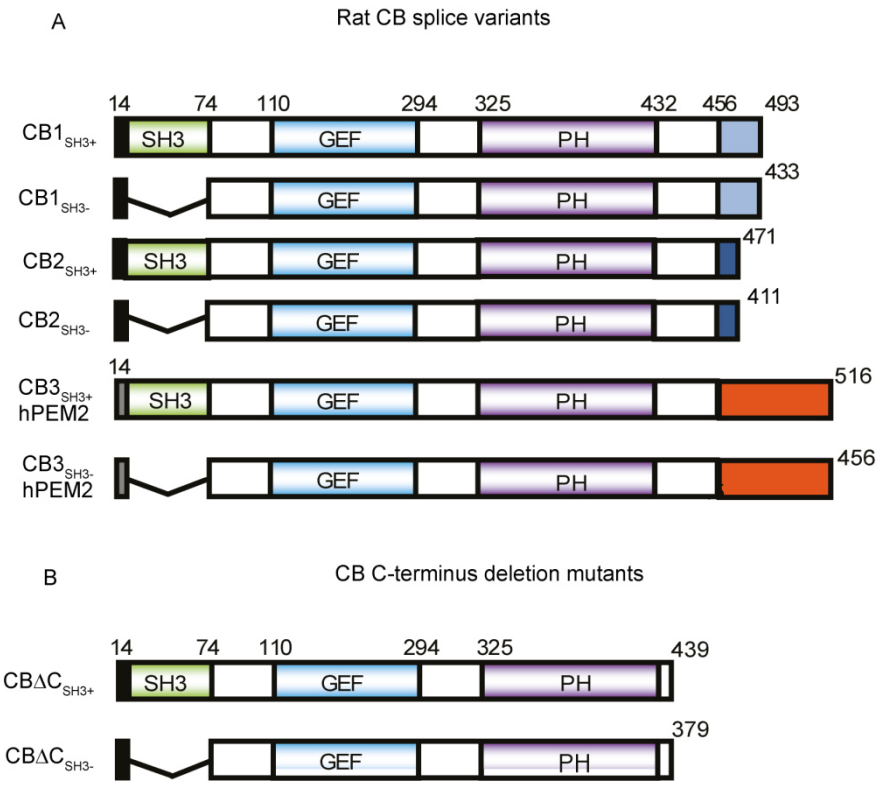
INTRODUCTION

Collybistin (CB; ArhGEF9) is a member of the Dbl family of guanine nucleotide exchange factors (RhoGEFs), known to regulate the GTPases Cdc42 (Reid et al., 1999) and TC-10 (Mayer et al., 2013) and contribute to the formation and stabilization of GABAergic synapses (Kins et al., 2000; Harvey et al., 2004; Chiou et al., 2011; Tyagarajan et al., 2011a). Targeted deletion of *ArhGEF9* revealed no symptom of body stiffness or increased sensory arousal, but rather caused enhanced anxiety along with impaired function of neuronal networks in the hippocampus, implying GABAergic, but not glycinergic, synapse dysfunction. Functional deficits in patients carrying *ARHGEF9* mutations affect GABAergic transmission (Kalscheuer et al., 2009).

ArhGEF9 encodes multiple CB splice variants (CB1-CB3), each differing only by a unique short C'-terminus sequence and by the presence or absence of an N-terminal src-homology 3 (SH3) domain (SH3+ or SH3-, respectively). All splice variants have a catalytic RhoGEF (or Dbl Homology; DH) domain and a pleckstrin homology (PH) domain (Harvey et al., 2004; Fritschy et al., 2012) (Suppl. Fig. 1). Mutations in hPEM2, human equivalent of CB3, have been reported in two cases of X-linked mental retardation without hyperekplexia, but associated with epilepsy, anxiety, and sensory hyperarousal (Kalscheuer et al., 2009; Marco et al., 2009). Structure analysis of CB bound to Cdc42 suggests that a competition with gephyrin (the main scaffolding protein of GABAergic synapses) might inhibit CB activation of Cdc42 via GDP/GTP exchange. In addition, the SH3 domain has also been implicated in CB conformation-induced function for gephyrin cell-surface translocation and postsynaptic clustering (Poulopoulos et al., 2009). However, given the lack of understanding of the significance of CB1-3 splice variants, current models of CB structure-function relationship may be premature.

It has become evident that GABAergic synapses are dynamic structures constantly adapting to changes in neuronal activity (Dobie and Craig, 2011; Chen et al., 2012; van Versendaal et al., 2012; Vlachos et al., 2013). We have proposed that dynamic regulation of the gephyrin scaffold at GABAergic synapses by various signaling cascades controlling gephyrin aggregation properties is a fundamental element of GABAergic synaptic plasticity (Tyagarajan and Fritschy, 2014). Here, we

demonstrate that various CB splice isoforms contribute to this process by differentially regulating gephyrin scaffold at distinct GABAergic synapses. In particular, we show that spatial segregation of various CB isoforms within neurons plays an important role in the modulation of gephyrin scaffolds. Therefore, our extensive characterization of four CB isoforms *in vivo* and *in vitro* offers insights into their regulation, to better understand their influence on gephyrin clustering. In particular, we show CB splicing regulation by activity-dependent signaling pathways and that CB isoforms exhibit different protein half-life due to ubiquitin-mediated regulation of their C'-terminus lysine residues. These results offer a mechanistic understanding of CB isoform regulation and reveal that distinct regulatory signals modulate specific CB isoforms to influence gephyrin scaffolding at GABAergic synapses.



Suppl. Figure 1: Protein domains of rat CB splice isoforms. (A) *ArhGEF9* expresses 6 different collybistin splice isoforms in neurons. Each of the CB1-3 isoform exists with or without the N-terminal SH3 domain. The C'-terminus sequence differences between CB1, CB2 and CB3 offer unique regulatory identity to each of the isoform. **(B)** Construction of the $CB\Delta C_{SH3+}$ and $CB\Delta C_{SH3-}$ mutants.

MATERIAL AND METHODS

Plasmids

CB isoforms $CB1_{SH3+}$, $CB1_{SH3-}$, $CB2_{SH3+}$ and $CB2_{SH3-}$ were cloned from rat whole brain RNA using primers Fwd: 5'-ATGCAGTGGATTAGAGGC-3'; Rev: 5'-CTAATAGTGCCATTTTCTTTGG-3' for CB1 isoforms, and Fwd: 5'-ATGCAGTGGATTAGAGGC-3'; Rev: 5'-CGCTAAGCTTCATGACTCTGCTGATCA-3' for CB2 isoforms. pCR3-V5-$CB1_{SH3+}$, pCR3-V5-$CB1_{SH3-}$, pCR3-V5-$CB2_{SH3+}$ and pCR3-V5-$CB2_{SH3-}$ were generated by sub-cloning the V5 tag into the pCR3-CB vectors using *HindIII* restriction site. The eGFP-gephyrin P1 variant has been described previously (Lardi-Studler et al., 2007). FLAG-gephyrin, FLAG-G, FLAG-GC and FLAG-E-domains were described previously (Tyagarajan et al., 2011b). pGEX2T-Cdc42 (Addgene plasmid 12969) has been described previously (Shinjo et al., 1990). One-STrEP-tag CB was created by sub-cloning the STrEP tag into pCR3-$CB1_{SH3+}$, $CB1_{SH3-}$, $CB2_{SH3+}$ or $CB2_{SH3-}$ vectors using *EcoR*I site. myc-$CB2_{SH3+}$ and myc-$CB2_{SH3-}$ have been described earlier (Tyagarajan et al., 2011a). All CB mutants were generated using site directed mutagenesis according to vendor manual (Agilent Technologies, USA) using pCR3-V5-CB splice isoforms as the template. The truncated V5-CBΔC_{SH3+} and V5-CBΔC_{SH3-} were generated by inserting a Stop codon after Ile 440 (Ile380 for CB_{SH3-}, respectively) into pCR3-V5-CB1 isoforms. HA-ubiquitin was a gift from Dr. Teier, Heidelberg, Gremany, pSUPERIOR-neo-GFP-CBShRNA146 was generated by Oligoengine using the sequence GATCGACGATGAGGAGGGAT.

Cell culture

Primary hippocampal neuron cultures were prepared as described previously (Buerli et al., 2007). Hippocampal cultures were transfected with eGFP-gephyrin and respective V5-CB construct with 1µg of total plasmid, using a combination of Lipofectamine 2000 (Life Technologies) and CombiMag (OZ Biosciences). The neurons were grown in 2 mL of growth media (Burli et al., 2007) for 7 days or 11 days prior to transfection. 1 mL of this conditioned media was transferred into a fresh 12-well dish prior to transfection. The plasmids were mixed in 30 µL of OptiMEM medium (Life Technologies), in a separate tube, master mix was prepared consisting of Lipofectamine 2000 (2 µL per sample) in OptiMEM (30 µL per sample). After incubating the Lipofectamine 2000 mix at room temperature for 5 min, 32 µL

of this mix was added to the tube containing the DNA. CombiMag was diluted (1:10) in OptiMEM media and 2 mL of the diluted CombiMag was added to each of the samples and mixed thoroughly. The transfection mix was incubated at room temperature for 15min before adding to the neurons. The 12-well dishes with the transfection reagents were placed over a magnetic plate inside the incubator. The transfection was stopped 25min later by transferring the coverslips into fresh 12-well dishes containing the conditioned media. The neurons were transfected after 8 or 11 days in vitro (DIV) and processed for immunofluorescence 7 days later (referred to as 8+7 DIV, respectively 11+7 DIV).

Primary mouse hippocampal neuron cultures were prepared similarly to the rat cultures using appropriate media. The dissociated cultures were plated initially in plating media (DMEM with high glucose, Glutamax, Pyruvate (Gibco 31966); Fungizone (final: 2.5 g/mL) Gibco; Gentamicin (final: 100ug/mL) Gibco; FCS: 10% Gibco). After 2h the media was changed to cell culture medium (Neurobasal, Gibco 21203-049; L-Glutamine (2mM) Gibco; Nu-Serum 10% (BD355500), B27 1x, Gibco). For each 18 mm poly-L-Lysine coated cover-slip, 50000 cells in 200 uL of plating medium was plated, and for a 6-well plate, 250000 cells in 3 mL plating medium was added to each well. The neurons were incubated at 37°C in 5% CO_2 and were transfected with appropriate plasmids similar to rat neurons.

HEK-293T cells were cultured at 37°C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). They were transfected with 2-3 µg DNA at 14-16h post plating using polyethylamine (PEI) according to the manufacturer's recommendation. The whole cell lysate was prepared 24h post transfection using EBC buffer containing Complete-mini (Roche) and phosphatase inhibitor cocktail (Sigma).

Ubiquitination assay

HEK-293T cells were transfected with 1 µg of V5-CB and HA-ubiquitin using PEI as per vendor suggestions. 16 h post-transfection, 5 µM MG132 (Tocris) was added and 5 h post-treatment, cells were lysed for denaturing Immunoprecipitation (IP). Cells were rinsed in cold PBS, scraped and transferred to chilled Eppendorf tubes. To pellet the cells, the tubes were centrifuged at 2000rpm for 5 min at 4°C and re-

solubilized in PBS containing 1% SDS, complete mini-protease inhibitor (Roche Diagnostics), phosphatase inhibitor cocktail 2 and 3 (Sigma–Aldrich), 5 mM dithiothreitol (DTT) (Sigma-Aldrich) and 20 mM iodoacetamide (Sigma-Aldrich). The cell membrane was disrupted by sonication using a nozzle sonicator at medium power for 20 s. After 10 min at room temperature, the lysate was re-naturated by adding chilled neutralization buffer containing 100 mM NaCl, 4.8 mM NaH_2PO_4, 5.2 mM Na_2HPO_4, 0.5% Nonidet P-40, 0.1% deoxycholic acid, 1 mM DTT, complete mini-protease inhibitor (Roche Diagnostics), phosphatase inhibitor cocktail 2 and 3 (Sigma–Aldrich) and 20 mM iodoacetamide. Cell debris was captured away by adding 20 µL sepharose beads and spun down for 15 min at maximum speed for 15 min at 4°C. The supernatant was stored at -80°C for further biochemical processing.

In vitro Complex formation

GST–Cdc42 and STrEP–gephyrin were expressed in bacterial strain BL21 pLyss Rosetta to an O.D. of 0.4 and induced with anhydrotetracycline (AHT), as recommended by the vendor (IBA, Germany) for 5 h. The bacterial cell pellet was re-suspended in EBC buffer containing the protease inhibitor and lysozyme, followed by sonication to disrupt the cells. The lysed cells were centrifuged at 18,000 rpm for 30 min and the supernatant was aliquoted and frozen at –80°C until further use. Fresh aliquots were used for all experiments, which were thawed and incubated with 20 µL of either glutathione beads (for GST-tagged proteins) and STrEP-Tactin beads (for STrEP–gephyrin) for 30 min on ice. These were then washed three times in EBC buffer and incubated with lysate of HEK-293 cells overexpressing the desired proteins with epitope tags for 60 min in a cold-room. The complexes were washed three times in EBC buffer before adding 2xSDS loading buffer and boiling the samples for SDS-PAGE and WB analysis.

Protein stability assay

V5-CB isoforms were transfected into HEK-293 T cells using the PEI protocol. 16 h after transfection 100 nM cyclohexamide dissolved in culturing medium was added and the cells were lysed in EBC buffer [50 mM Tris- Cl pH 8.0, 120 mM NaCl and 0.5% Nonidet P-40) containing complete mini-protease inhibitor (Roche Diagnostics) and phosphatase inhibitor cocktail 2 and 3 (Sigma–Aldrich)] after 0, 1, 2, 4 or 8 h of treatment for 20 min at 4°C. Cell debris was pelleted by spinning at maximum speed

for 15 min at 4°C, the supernatant was boiled in 5x sample buffer containing DTT for 10 min at 72°C and loaded on 10% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad) and run at 120 V in Tris-glycine SDS running buffer at room temperature. After WB the protein-band intensity was measured using LI-COR odyssey scanner and image studio. The area under the curve was normalized to actin and a half-life curve was fitted using Graphpad Prism.

Immunoprecipitation and western blot analysis

For immuneprecipitation 0.8 µL anti V5 antibody was added to the cell lysate and incubated over-night on a rotating wheel at 4°C and the protein complex was precipitated by using 50 µL of Protein A and Protein G Plus-Agarose beads (Calbiochem) in EBC buffer. The beads were washed once in EBC-based high-salt buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl and 1% Nonidet P-40 (Sigma-Aldrich) and twice in EBC buffer. After boiling the samples in 2xSDS sample buffer containing 15% 2-Mercaptoethanol (Bio-Rad) for 10 min at 72°C the supernatant was loaded onto SDS-polyacrylamide gels and run at 140V at room temperature. After transferring the protein bands onto PVDF membranes with constant 35 mA in Tris-glycine transfer buffer, WB were performed by blocking the membranes with 5% western blocking reagent (Roche Diagnostic) in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and later incubating with primary antibody overnight at 4°C. Secondary antibody coupled to horseradish peroxidase or IRDye® (LI-COR) was used to visualize the protein bands.

RNA isolation and quantitative realtime-PCR

RNA was extracted from rat hippocampus or cortex or from cultured cortical neurons using Sigma-Aldrich GenElute™ Mammalian Total RNA Miniprep Kit; cDNA was prepared using random hexamers and SuperScript® III Reverse Transcriptase (invitrogen). Quantitative PCR was performed on a 7900 HT Fast Real Time PCR system (Applied Biosystems). 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Biodyne) was used with the designed primers (Table 1) to amplify the mRNA. Changes in mRNA levels were calculated using the ΔΔCt Method (Pfaffl, 2004) and were normalized to that of *Gapdh* mRNA. Bar charts and statistics were performed using Graphpad Prism. Two way ANOVA with Bonferroni post-hoc correction with the following significances: $P<0.001$ ***; $P<0.01$ **; $P < 0.05$ *.

Table 1 qPCR primer

CB1 fwd	5'-GTAGGGTTGGAGAGGAAGAG-3'	CB1 rev	5'-TTGTGGTGGATAGGAAGGTG-3'
CB2 fwd	5'-GCACAACAAGGAAACCGAAGA-3'	CB2 rev	5'-TGGGTTACTTTCTGTTAGACGCTTT-3'
CB3 fwd	5'-GTTAACCCCCTTCAAAAAA-3'	CB3 rev	5'-TTATATCCTTCCACAATAC-3'
SH3 fwd	5'-TGGTTTCCTGCCAGCTTTGT-3'	SH3 rev	5'-TCTGGTCCCGGTTCTGTAGTG-3'
GABRA2 fwd	5'-ATCCAGGATGACGGAACATTG-3'	GABRA2 rev	5'-GGAAAGTCCTCCAAGTGCATTG-3'
panCB fwd	5'- CCCTGCTTCTTGGAGCATCA -3'	panCB rev	5'- TGATAGCGGCTGTCCTTCATC -3'
GAPDH fwd	5'-CATGGCCTTCCGTGTTCCTA-3'	GAPDH rev	5'-CCTGCTTCACCACCTTCTTGA-3'

Antibodies and immunohistochemistry

Guinea pig anti-synapsin-1/2 antibody (1:1000, Synaptic Systems, Göttingen, Germany), Mouse anti-V5 antibody (1:5000, Invitrogen, Carlsbad, USA), mouse anti-V5 antibody (1:3000, Acris, SanDiego, USA), rabbit anti-VGLUT antibody (1:10,000, Synaptic Systems, Göttingen, Germany), rabbit anti-VGAT antibody (1:3000, Synaptic Systems, Göttingen, Germany), mouse anti-actin (C4 clone) antibody (1:20'000, EMD Millipore Corporation, Billerica, USA), mouse anti-gephyrin antibody (mAb7a, 1:3000; or 3B11, 1:10,000; Synaptic Systems, Göttingen, Germany), mouse anti-FLAG (1:5000, Sigma, Saint Louis, USA), STrEP TACTIN HRP (1:30,000), STrEP-tag Purification (IBA GmbH, Göttingen, Germany), mouse monoclonal anti-PanCB was raised against the peptide sequence LGRPLQNRDQ (Abmart, Shanghai, China).

Cells were fixed for 10 min in 4% PFA, rinsed in PBS and permeabilized with 0.01% Triton X-100 containing 10% normal goat serum. Immunohistochemistry was performed by incubating the cells with the primary antibodies diluted in PBS containing 10% normal goat serum for 60 min. After washing in PBS the cells were incubated with the secondary antibodies coupled to Cy3 or Cy5 (1:1000, Jackson ImmunoResearch) for 30 min. After drying the cells were mounted with fluorescent mounting medium (Dako Cytomation, Carpinteria, CA). Everything was performed at room temperature.

Image analysis and quantification

All images were acquired using confocal laser-scanning microscopy (LSM 510 Meta or LSM 710, Carl Zeiss, Jena, Germany), using a 100x or 63x lens (NA 1.4). The

pinhole was set for all channels at 1 Airy unit and pixel size was 130 nm. For each condition 12 cells from 3 independent batches were imaged by acquiring a z-stack (3-4 steps at 0.5 µm). Image analysis was performed using ImageJ (http://rsb.info.nih.gov/ij/). On each cell a dendritic segment was lined out for analysis and maximum intensity projection was used. Threshold was kept the same throughout the analysis. For detailed protocol see (Tyagarajan et al., 2011a).

Fluorescent in-situ hybridization (FISH)

Primary hippocampal cultures were prepared and transfected as described earlier (Tyagarajan et al., 2011a). In-situ hybridization of high resolution was performed with QuantiGene ViewRNA kit from Panomics (Affimetrix) using customized probes designed and provided by the company according to our labeling choice. The transfected or untransfected hippocampal neurons were briefly fixed using 4% paraformaldehyde (PFA) solution for 30min at room temperature (RT). The probe hybridization and amplification was performed as described in the user manual. All images were acquired using laser scanning microscope (LSM710, Zeiss) and images were processed using ImageJ.

RESULTS

Collybistin acts downstream of GABAA receptors to regulate gephyrin clustering

It has been shown that gephyrin clustering is disrupted by the targeted deletion of the $GABA_AR$ α2 or γ2 subunits (Essrich et al., 1998; Panzanelli et al., 2011). To directly demonstrate the importance of CB for clustering of gephyrin at GABAergic synapses, we first investigated the molecular basis for gephyrin cluster disruption.

We measured gephyrin and CB transcripts by qRT-PCR analysis in total brain RNA from 3-month-old WT (C57BL6) and α2-KO (C57BL6) mice (in which gephyrin postsynaptic clustering is severely impaired) (Panzanelli et al., 2011). No difference was found in the mRNA levels of *ArhGEF9* and *GPHN* gene products (Fig. 1A). Next, we examined the protein levels of CB and gephyrin by Western blotting (WB) using whole brain lysates from 3-month-old WT and α2-KO mice. In order to detect the endogenous CB protein, we generated in-house mouse monoclonal PanCB antibody (Abmart) and confirmed its specificity using mouse and rat whole brain extracts. This PanCB antibody recognized two main CB splice isoforms (CB_{SH3+} and CB_{SH3-}) and the CB-KO brain homogenates served as a negative control (Fig. 1B); furthermore we tested its specificity using overexpression heterologous system HEK-293 cells transfected with V5-CB isoforms. The α2-KO mice showed reduced levels of gephyrin and CB protein (Fig. 1 C-D), suggesting that CB protein levels might be the rate-limiting step for gephyrin postsynaptic clustering. Therefore, we tested whether overexpression of CB can rescue gephyrin clustering by transfecting CB cDNA into cultured neurons. We performed these experiments using hippocampal neuron cultures prepared from γ2-KO mice, in which gephyrin clustering is totally abolished (Essrich et al., 1998). We co-transfected eGFP-gephyrin and myc-$CB2_{SH3+}$ or myc-$CB2_{SH3-}$ at 8 days *in vitro* (DIV 8) and analyzed for the presence of eGFP-gephyrin clusters 4 days post-transfection (DIV 8+4). Overexpression of either of the CB2 isoforms rescued eGFP-gephyrin clustering in the γ2-KO neurons, demonstrating the efficacy of CB for rescuing gephyrin clustering in the absence of synaptic $GABA_ARs$; furthermore, emphasizing the importance of CB for postsynaptic gephyrin stabilization. Interestingly, we found isoform-specific morphological differences between gephyrin clustering in neurons

co-transfected with myc-CB2$_{SH3+}$ or myc-CB2$_{SH3-}$. For instance, there were many smaller (Fig. 1; white arrows) but fewer larger (Fig. 1; white arrowhead) eGFP-gephyrin clusters in neurons expressing CB2$_{SH3+}$ isoform. On the other hand, neurons co-transfected with CB2$_{SH3-}$ isoform contained more large clusters and fewer small clusters, suggesting that CB isoforms influence gephyrin scaffolding differentially.

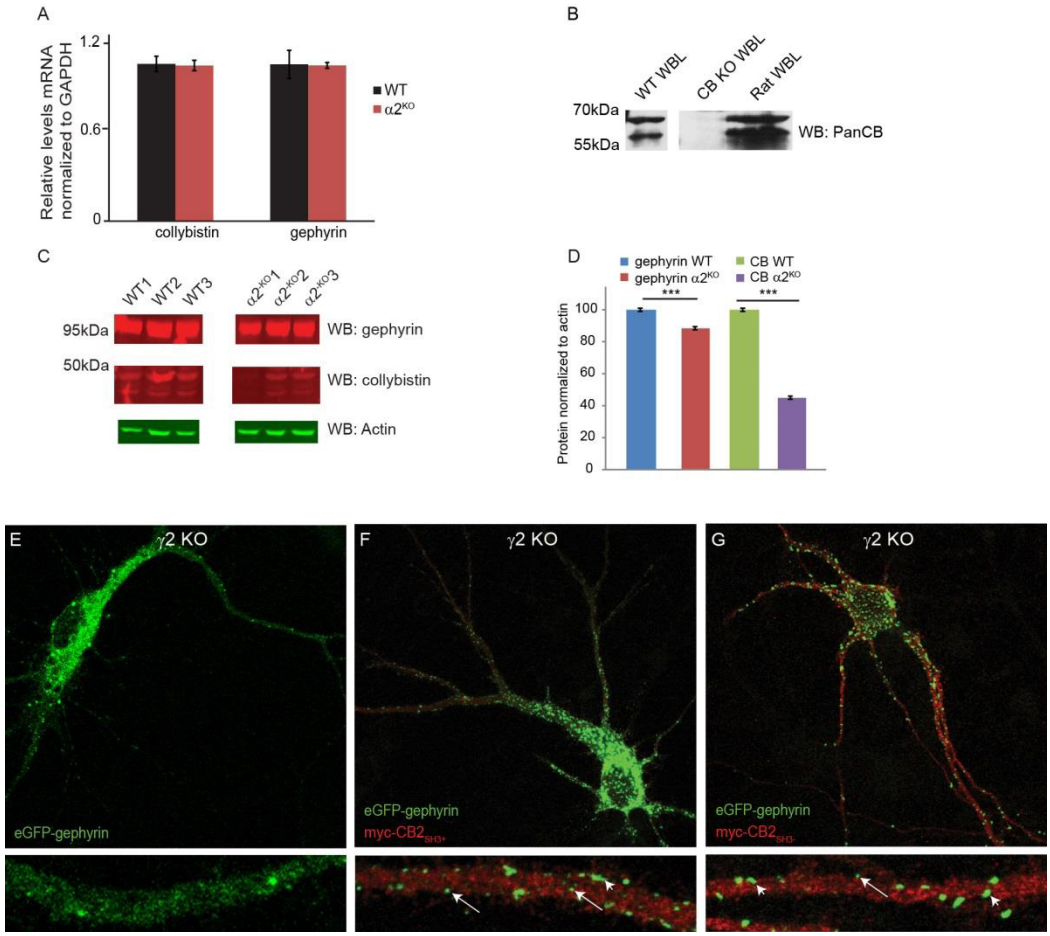


Figure 1: Gephyrin clustering in GABA$_A$R deficient mice is rescued by CB expression. (A) qRT-PCR analysis of gephyrin and CB mRNA levels in WT and α2-KO mice. **(B)** Characterization of the mouse PanCB antibody using C57BL6 mouse (WT), ArhGEF9-KO mouse (CB-KO) and Wistar rat brain homogenates. **(C)** WB analysis for gephyrin (top panel), CB (middle panel) and actin (bottom panel) to determine protein levels in WT and α2-KO mice. **(D)** Quantification of the protein bands for gephyrin and CB after normalization to actin loading control. Represented as fold change with SEM between samples. Unpaired t-test, P <0.0001. **(E)** γ2-KO mouse E18 cultured neuron transfected with eGFP-gephyrin. **(F)** γ2-KO cultured neuron co-transfected with eGFP-gephyrin and myc-CB2$_{SH3+}$. **(G)** γ2-KO cultured neuron co-transfected with eGFP-gephyrin and myc-CB2$_{SH3-}$. Arrows indicate small eGFP-gephyrin clusters and the arrowhead large eGFP-gephyrin clusters.

Neuronal activity modulates CB splice site selection in neurons

There is accumulating literature showing that gephyrin clusters at GABAergic synapses are highly dynamic in nature. Hence we examined whether CB transcription and/or post-transcriptional regulation could be mechanisms for gephyrin clustering dynamics in response to changes in neuronal activity. In order to test this possibility, we treated cultured neurons with KCl (30 mM) 90 min and examined the changes in CB splicing using qRT-PCR. To ensure that neuronal depolarization affected *ArhGEF9* transcript splicing but no other transcripts associated with inhibitory synapses, we tested for changes in α2-$GABA_AR$ subunit expression levels. We normalized the *ArhGEF9* and *Gabra2* mRNA levels to the house keeping gene GAPDH transcripts (Fig. 2A), and compared changes relative to the mock-treated controls. Prolonged neuronal depolarization using KCl resulted in significant reduction in the levels of CB1, CB3 and SH3 splice cassette mRNA expression levels. However, the expression of CB2 and α2 subunit mRNA was unaffected; suggesting neuronal activity specifically regulates CB1 and CB3 splicing.

Next, we investigated which signaling cascade might influence CB1 and CB3 splice site selection after neuronal depolarization. We blocked signaling cascades that are typically activated upon neuronal activity (MAPK, PKC, CaMK and cAMP) using specific pharmacological inhibitors and analyzed for changes in CB mRNA isoform splicing (Fig. 2B). Inhibition of CaMK signaling pathway in the presence of KCl specifically affected CB1 and CB3 splicing, while inhibitors of MAPK, cAMP, PKC pathways had no influence on CB splicing. We eliminated the possibility for transcriptional changes in CB transcript levels after neuronal depolarization by quantifying total CB transcripts using PanCB probe and normalizing it to GAPDH levels.

CB isoform-specific affinity differences for binding partners

Identification of activity-dependent CB splicing regulation prompted us to examine whether biochemical interaction differences would functionally segregate these CB isoforms. Considering that CB was initially identified as a gephyrin-interacting protein (Kins et al., 2000), we decided to test biochemical affinity differences between CB1 and CB2 isoforms with gephyrin. In order to achieve this goal, we co-transfected FLAG-gephyrin and V5-$CB1_{SH3+}$, V5-$CB1_{SH3-}$, V5$CB2_{SH3+}$ or V5-$CB2_{SH3-}$

isoforms into HEK-293 cells. IP for V5-CB followed by WB for FLAG showed an interaction between all four V5-CB isoforms tested and FLAG-gephyrin (Fig. 3A-B); however, we consistently found the $CB1_{SH3+}$ interaction with FLAG-gephyrin to be the weakest, and the $CB2_{SH3-}$ interaction to be the strongest. We excluded the possibility that this binding difference is due to protein expression variations between samples by checking for the protein expression levels in all our samples (Fig. 3A'-B').

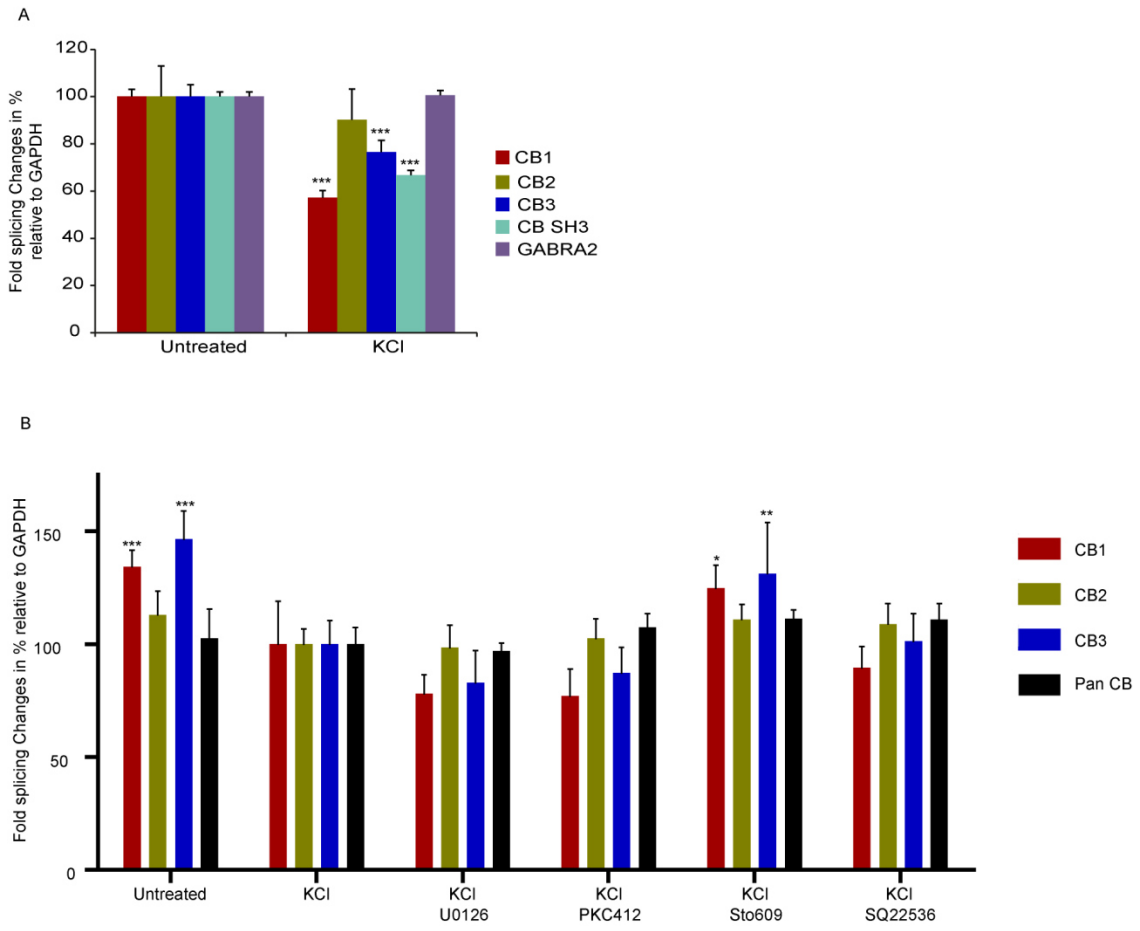


Figure 2: Neuronal activity dependent CB splicing *in vitro*. (A) qRT-PCR from DIV18 cortical neurons treated with KCl (30mM, 90min) show a decrease in CB1, CB3 and SH3 domain containing CB mRNA levels, CB2 and *GABRA2* transcript levels are not altered. **(B)** Blocking of immediate-early signaling (MAPK (U0126, 10 µM), PKC (PKC412, 10 µM), CaMK (Sto609, 5 µM) and cAMP (SQ22536, 200 µM) in the presence of KCl (30mM) shows CaMK pathway specifically regulates CB1 and CB3 splicing. One-way ANOVA, $p<0.001$.

A known substrate for CB is Cdc42, recently TC-10 was also described as a novel CB substrate (Mayer et al., 2013). Hence, we next tested for CB isoform interaction with Cdc42 *in vitro* using GST-Cdc42 and V5-CB isoforms. Interaction of V5-CB1$_{SH3+}$, V5-CB1$_{SH3-}$, V5-CB2$_{SH3+}$ or V5-CB2$_{SH3-}$ over-expressed in HEK-293 cells with bacterial overexpressed GST-Cdc42 was tested in the presence of γsGTP or GDP (Suppl. Fig. 2A-B). Consistent with our early observations, V5-CB1$_{SH3+}$ isoform showed the weakest interaction while V5-CB2$_{SH3-}$ isoform showed the strongest affinity, suggesting CB2 isoforms could have better affinity for binding partners.

We have demonstrated earlier that myc-CB2$_{SH3+}$ and myc-CB2$_{SH3-}$ can form ternary complex with gephyrin and Cdc42 *in vitro*, with the latter being stronger than the former (Tyagarajan et al., 2011a). Hence, we wanted to also analyze whether V5-CB1$_{SH3+}$ and V5-CB1$_{SH3-}$ isoforms can form ternary complexes with gephyrin and Cdc42 to the same extent as V5-CB2$_{SH3+}$ and V5-CB2$_{SH3-}$ (Fig. 3C-F). Interestingly, we could not detect a ternary complex between V5-CB1$_{SH3+}$ and V5-CB1$_{SH3-}$ when Cdc42 was incubated with γsGTP or GDP (Fig. 3C-D). However, we could reproduce our earlier finding and demonstrate that V5-CB2$_{SH3-}$ forms a ternary complex with FLAG-gephyrin and γsGTP-Cdc42 and a weaker complex with GDP-Cdc42 (Fig. 3D-E). These biochemical differences between CB1 and CB2 isoforms suggested that C'-terminus sequence differences (Suppl. Fig 1A) influence the overall CB1 structure and its affinity towards its binding partners.

The ternary complex between CB-Cdc42–gephyrin is hard to explain on the basis of the single binding site for both gephyrin and Cdc42 in the DH domain of CB. We speculated that homo- and hetero-dimerization of CB isoforms could provide for multiple CB interaction sites within the same complex. We tested this hypothesis by immobilizing STrEP-CB1$_{SH3-}$ or STrEP-CB2$_{SH3+}$ overexpressed in HEK-293 cellsusing STrEP-tactin beads, and incubating these beads with extracts of HEK-293 cells containing overexpressed V5-CB1$_{SH3+}$, V5-CB1$_{SH3-}$, V5-CB2$_{SH3+}$ or V5-CB2$_{SH3-}$ isoforms (Fig. 3G). We determined that V5-CB specifically interacts with STrEP-CB isoforms to form homo- and hetero-dimers *in vitro,* using WB against V5 tag (Fig. 3G; Suppl. Fig2C). The negative control using STrEP-tactin beads did not show any interaction with V5-CB2$_{SH3-}$ or V5-CB2$_{SH3+}$ (Fig. 3G; lane1), suggesting that the observed

dimerization is specific, indeed. The loading controls for the expression levels of STrEP-CB and V5-CB isoforms are shown (Fig. 3G, lower panels).

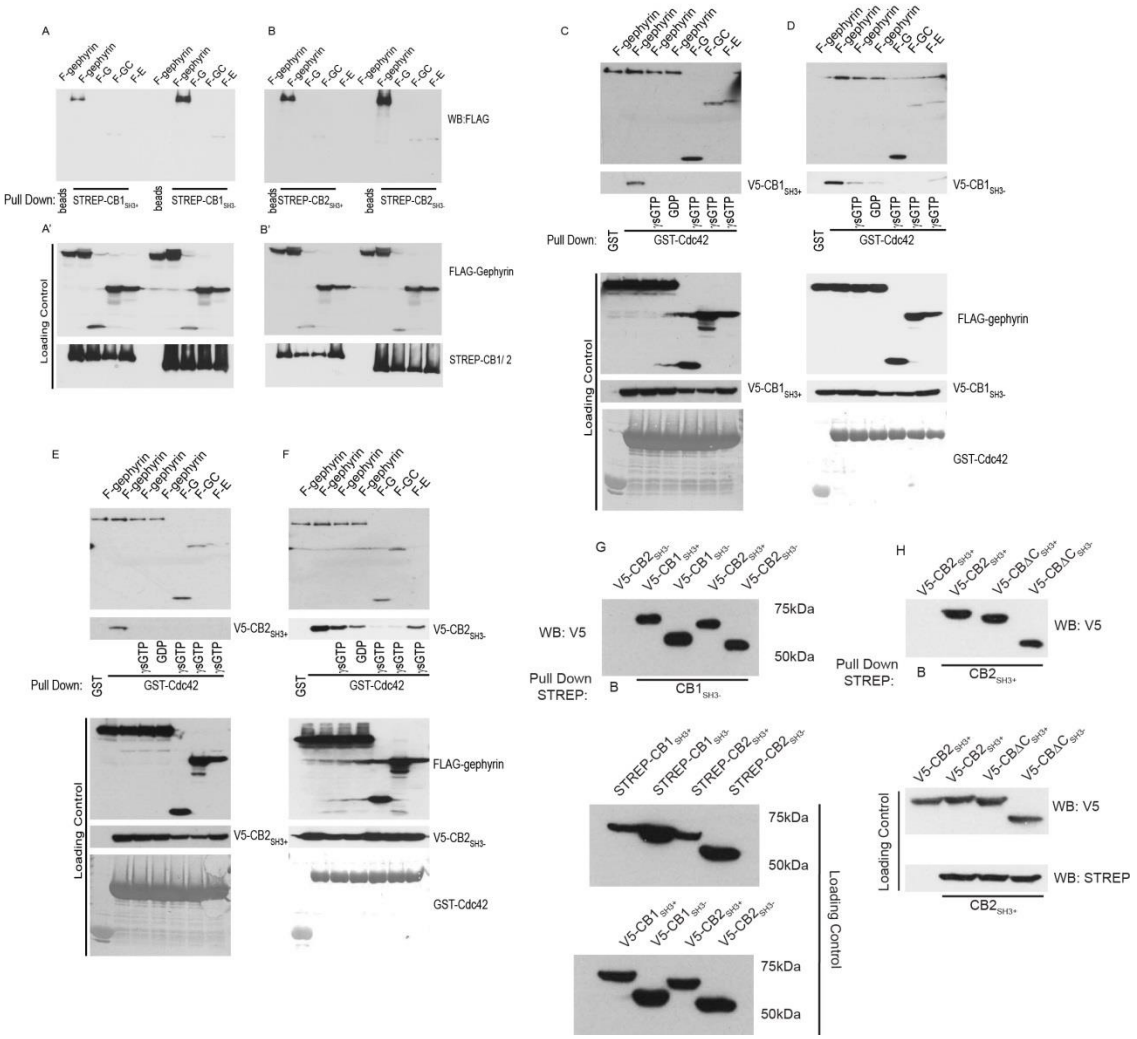
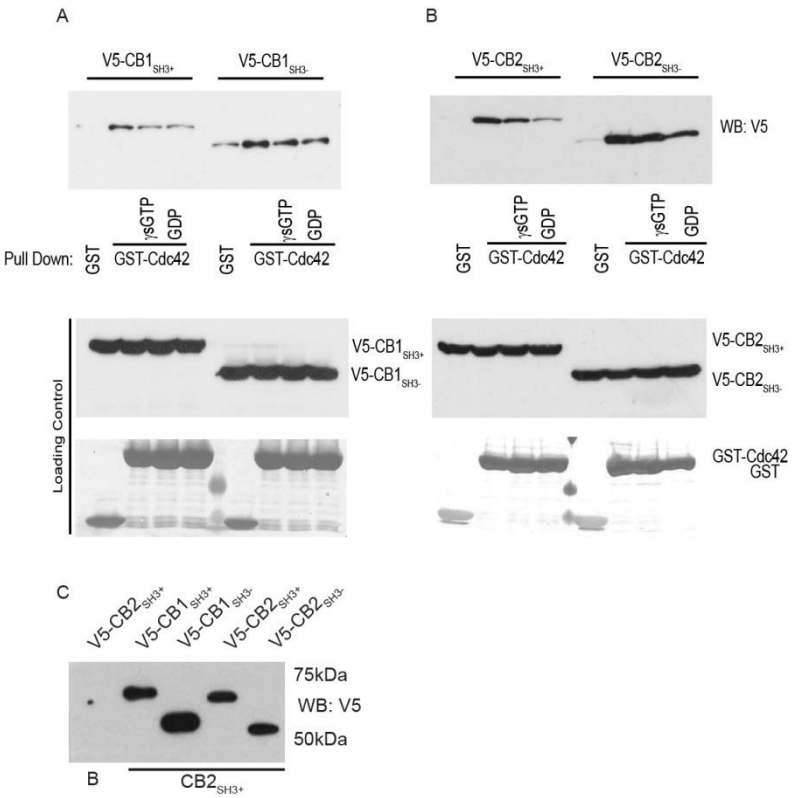


Figure 3: Biochemical affinity differences between CB1 and CB2 isoforms. (A-B) Interaction between STrEP-CB1$_{SH3+}$, CB1$_{SH3-}$, CB2$_{SH3+}$ and CB2$_{SH3-}$ isoforms with FLAG-gephyrin. Pull down for STrEP-CB using STrEP-tactin beads and WB for FLAG-gephyrin using anti-FLAG antibody shows strong interaction with SH3 lacking isoforms. The protein loading controls are shown in the panel below. **(C-D)** Complex formation between V5-CB1$_{SH3+}$ or CB1$_{SH3-}$ with FLAG-gephyrin and GST-Cdc42 in the presence of GDP or γsGTP. **(E-F)** Complex formation between V5-CB1$_{SH3-}$ and V5-CB2$_{SH3-}$ with gephyrin and Cdc42. Note: Only full length FLAG–gephyrin (lane 2,3,4), and not the single domains (FLAG-G, FLAG-GC or FLAG-E)(lane 5,6,7) complex with GST-Cdc42 in the presence of V5-CB. **(G)** Homo- and hetero-dimerization of STrEP-CB1$_{SH3-}$ with V5-CB1 and V5-CB2 isoforms. **(H)** Protein-protein interaction of STrEP-CB2$_{SH3+}$ with V5-CBΔC$_{SH3+}$ and V5-CBΔC$_{SH3-}$. The protein loading controls are shown in the lower panels.

The C'-terminus of CB contains a coiled-coil (CC) domain (Kins et al., 2000) (www.expasy.org), and it is well established that CC domains facilitate protein oligomerization. Hence, we wanted to test whether the C'-terminus of CB was essential for this inter- and intra-molecular interaction. We generated V5-CBΔC_{SH3+} and V5-CBΔC_{SH3-} mutants and incubated them with immobilized STrEP-$CB2_{SH3+}$ onto STrEP-tactin beads. HEK-293 cell lysate containing overexpressed V5-$CB2_{SH3+}$ served as positive and negative controls; WB against V5 tag showed interaction between STrEP-$CB2_{SH3+}$ and V5-$CB2_{SH3+}$ (positive control), and also with V5-CBΔC_{SH3+} and V5-CBΔC_{SH3-} (Fig. 3H, lanes2-4). Protein loading controls are also shown (Fig. 3H, bottom panels). These results confirm CB homo/heterodimerization and demonstrate that this phenomenon is independent of the C'-terminus sequence (and hence of the CC domain).



Suppl. Figure 2: Biochemical characterization of CB interactions. (A-B) Interaction between V5-CB isoforms and GST-Cdc42. Pull down for GST-Cdc42 and WB for V5 after incubating beads containing GST-Cdc42 with GDP or γsGTP shows strong interaction between the SH3-lacking CB isoforms and Cdc42. Pull-down of V5-CB was made after incubating beads containing GST-Cdc42 with GDP or GTPγS. The protein loading levels are shown in the bottom panels. **(C)** Homo- and hetero-dimerization of STrEP-$CB2_{SH3+}$ with V5-CB isoforms. The protein loading controls are shown in the lower panels.

CB splice isoforms exhibit differential protein half-life.

The observed activity-dependent modulation of CB1 mRNA splicing and not CB2 mRNA, followed by biochemical binding differences between CB1 and CB2 suggested that CB1 isoform could have a more dynamic protein stability. In order to test this we compared the protein half-life of CB1 and CB2 splice isoforms, using heterologous expression in HEK-293 cells. V5-CB1$_{SH3+}$, V5-CB1$_{SH3-}$, V5-CB2$_{SH3+}$ or V5-CB2$_{SH3-}$ cDNA constructs were transfected into HEK-293 cells and 14-16 h post-transfection cells were treated with cyclohexamide (100 nM) to block protein translation. The extracts were prepared at various time-points thereafter and protein expression levels were determined using WB (Fig. 4A-D, bottom panel). The half-life of V5-CB1$_{SH3+}$, V5-CB1$_{SH3-}$, V5-CB2$_{SH3+}$ and V5-CB2$_{SH3-}$ was calculated after normalizing to actin levels within individual samples (Fig. 4A-D). As predicted initially CB1 isoforms exhibited shorter half-life compared to their CB2 counterparts. CB1$_{SH3-}$ exhibited the shortest half-life of less than 2 h, while CB2$_{SH3-}$ exhibited the longest half-life of more than 7 h (regression analysis with fitted R values are shown). The CB1$_{SH3+}$ and CB2$_{SH3+}$ seem to have an intermediate half-life around 3-4 h. These data point to the importance of both SH3 domain and C'-terminal sequence in determining the protein half-life.

To confirm the importance of C'-terminal sequence for isoform-specific protein half-life we tested the protein half-life of the C'-terminal deletion mutants V5-CBΔC$_{SH3+}$ and V5-CBΔC$_{SH3-}$ in HEK-293 cells. Deletion of the C'-terminal sequence stabilized the CB proteins, with V5-CBΔC$_{SH3-}$ showing an intermediate stability compared to V5-CB1$_{SH3-}$ and V5-CB2$_{SH3-}$ (Fig. 4E) and V5-CBΔC$_{SH3+}$ exhibiting a stability very similar to that of V5-CB2$_{SH3+}$ (Fig. 4F). This result confirmed the influence of both the SH3 domain and the C'-terminus on CB protein stability and suggested distinct regulation differentiates CB1 and CB2 isoforms.

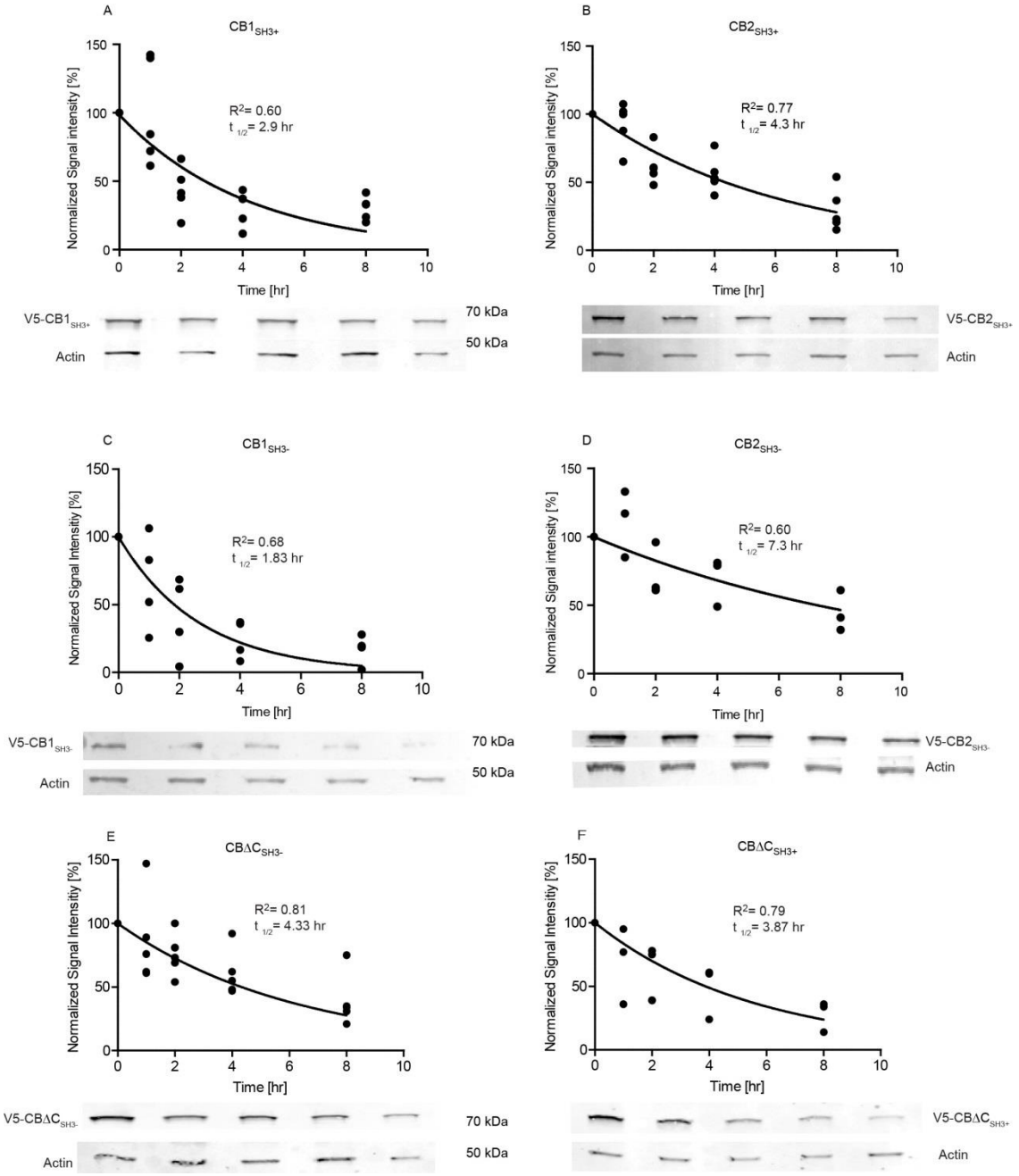


Figure 4: Protein half-life of V5-CB1 and V5-CB2 splice isoforms. (A) Protein stability of V5-$CB1_{SH3+}$ was determined by collecting the protein samples from HEK-293 cells at 0, 1, 2, 4 and 8 h in the presence of cyclohexamide (CHX) (0.1mM) and protein signal intensity relative to actin was plotted. The $t_{1/2}$ and R^2 values were determined (inset). **(B)** Protein half-life of $CB2_{SH3+}$ isoform. **(C)** Protein half-life of $CB1_{SH3-}$ isoform. **(D)** Protein half-life of $CB2_{SH3-}$ isoform.. $CB1_{SH3-}$ shows the shortest half-life (1.8 h), while $CB2_{SH3-}$ has the longest half-life (7.3 h). **(E)** Protein half-life of V5-$CB\Delta C_{SH3-}$ is stabilized compared to V5-$CB1_{SH3-}$ and V5-$CB2_{SH3-}$. **(F)** Protein half-life of V5-$CB\Delta C_{SH3+}$ is also longer compared to V5-$CB1_{SH3+}$ and V5-$CB2_{SH3+}$.

C'-terminus regulation determines the stability of CB1 and CB2 protein isoforms.

Isoform-specific protein stability differences provide a mechanism for functional differences at synapses to dynamically modulate gephyrin clustering. Hence, we wanted to determine the underlying basis for CB isoform-specific protein half-life differences. We co-transfected HEK-293 cells with V5-CB1 and V5-CB2 isoforms along with HA-ubiquitin and blocked protein degradation using the proteasome inhibitor MG132 (5 μM, 8 h). Immunoprecipitation of V5-CB was performed under mild denaturing conditions to make sure that only the covalently conjugated ubiquitin is detected in our assay. WB for HA-ubiquitin showed ubiquitination bands, especially in lanes treated with MG132 (Fig. 5A, lanes 2, 4, 6 and 8). Probing for V5-CB and HA-ubiquitin levels using WB against V5 or HA epitope tags showed equal expression levels (Fig. 5A'). Once CB ubiquitination was established, we performed a bioinformatics analysis using online databases (http://bdmpub.biocuckoo.org/; http://www.ubpred.org/) to identify ubiquitination sites on CB1 and CB2 isoforms. We identified the C'-terminal lysine residues on $CB1_{SH3+}$ (K491/K492) and $CB1_{SH3-}$ (K431/K432) as highly probable ubiquitination sites (Fig. 5B). We systematically mutated the selected lysine residues to prevent their ubiquitination and determined the effects on the half-life of the mutant proteins. Mutating the predicted lysine residues on CB1 isoforms significantly increased the half-life of both $CB1_{SH3+}$ and $CB1_{SH3-}$ (Fig. 5C-D).

Next, we wanted to identify the lysine residues that regulate CB2 isoforms protein stability. Initial analysis of C'-terminus lysine residues (K458, K461, K463) showed no effect on the protein half-life (data not shown). However, when we generated the mutant K468R ($CB2_{SH3+}$) and K408R ($CB2_{SH3-}$) and tested their protein half-life we found that the protein half-life was further reduced (Fig. 5E-F). This unexpected finding suggested that K468 and K408 residues in CB2 isoforms are important regulatory sites that facilitate ubiquitination at another unidentified residue(s).

We then selected lysine residues with next highest scores for ubiquitination on $CB2_{SH3+}$ and mutated them to analyze the protein half-life. Interestingly, K439R mutation significantly increased the protein half-life and K432R mutation significantly reduced the protein half-life of $CB2_{SH3+}$.

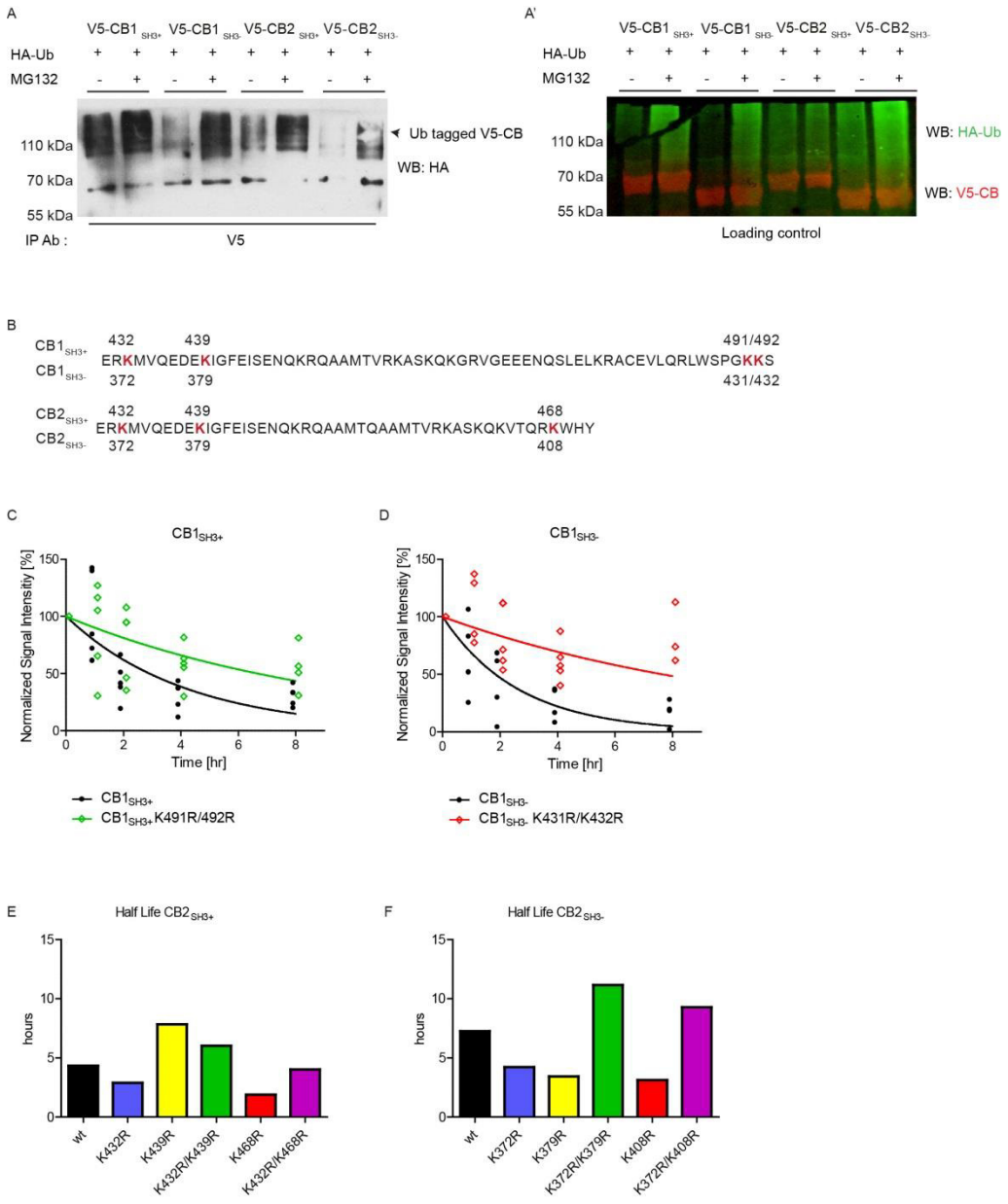


Figure 5: CB protein stability is determined by specific C'-terminus Lys residues. (A) HEK-293 cells co-expressing HA-ubiquitin and V5-CB1 and CB2 isoforms treated with MG132 (5 μM), IP for V5-CB and WB for HA allowed us to determine CB ubiquitin conjugation. **(A')** The IP'ed levels of V5-CB isoforms are shown after probing with V5 antibody. **(A")** Protein expression levels of HA-Ub (green) and V5-CB isoforms (red) in HEK-293 cells. **(B)** Location of mutated ubiquitination sites on CB1 and CB2 C'-terminal sequence. **(C-D)** The predicted K491R/K492R ubiquitin residues in CB1 were mutated Lys-Arg and the protein stability of the mutants was determined in HEK 293 cells. The ubiquitin site mutants have a longer half-life compared to the WT counterparts. **(E)** Protein half-life of V5-$CB2_{SH3+}$ C'-terminus ubiquitin site mutations was compared to identify their regulatory effect. **(F)** Half-life of the V5-$CB2_{SH3-}$ C'-terminus lysine mutants was compared to determine their effect on protein stability.

When we generated and tested the double mutant K432R/K468R, we found that this double mutant was more stable compared to the individual mutants and had a half-life similar to the WT protein. We wondered whether the K439 residue was the key residue regulating $CB2_{SH3+}$ protein stability, and generated the double mutant K432R/K439R to test this possibility. This double mutant exhibited a greater protein stability, confirming that K439 is indeed the critical determinant for $CB2_{SH3+}$ half-life (Fig. 5E).

For identifying ubiquitination sites on $CB2_{SH3-}$ we focused on the K379 residue, as it is similar to K439 on $CB2_{SH3+}$. However, upon mutating K379 we found that $CB2_{SH3-}$ protein stability was further reduced, which is opposite to the effect of K439 mutation on $CB2_{SH3+}$. To understand this phenomenon better, we generated the double mutant K372R/K379R, disrupting two neighboring lysine residues. The protein half-life of this double mutant was significantly increased compared to the WT control or their individual mutations (K372R or K379R) (Fig. 5F). Our data suggest that K379, although an important regulator of $CB2_{SH3-}$ protein stability, acts in tandem with both K372 and K408 residues. In order to confirm this idea, we generated K372R/K408R double mutant and found that this combination stabilized $CB2_{SH3-}$ (Fig. 5F). The antagonistic effects of K372 and K379 residues on $CB2_{SH3+}$ and $CB2_{SH3-}$ half-life add weight to the idea that regulatory mechanisms between isoforms are distinct; this, in turn, is determined by the overall protein conformation.

CB1 and CB2 isoforms enhance gephyrin clustering is neurons.

Given our observations that CB1 and CB2 isoforms differ in their mRNA and protein regulation, we wanted to determine their influence on eGFP-gephyrin synaptic clustering in primary hippocampal neurons. For this purpose, we transfected neurons at 8 DIV and analyzed them at DIV 8+7. Representative images of control eGFP-gephyrin (green) and CB (blue) co-transfected neurons are shown (Fig. 6A-E). Synaptic localization was determined by eGFP-gephyrin apposition to VGAT/VGLUT positive terminals (magenta). The enlarged segment of a dendrite shows the synaptic co-localization of eGFP-gephyrin clusters (Fig. 6A-E, lower panels). Co-localization of the gephyrin puncta with the $GABA_AR$ γ2 subunit was also assessed (Fig. 6F-K').

Quantitative analysis (one-way ANOVA; density $F_{4,78}$= 19.6; p<0.05) did not show any significant difference between CB1 and CB2 isoforms in the density of synaptic eGFP-gephyrin clusters (Bonferroni post-hoc test; p=0.85) (Fig. 6L).

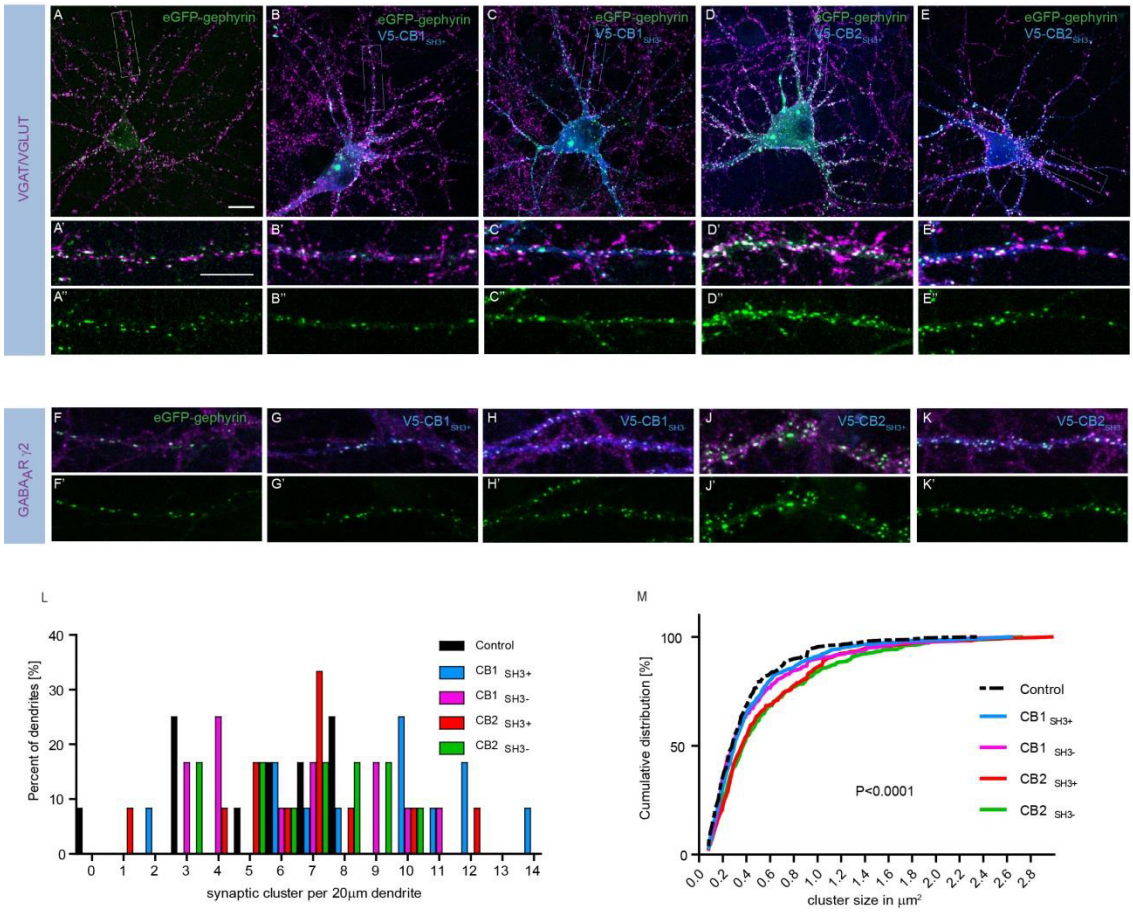


Figure 6: Morphological changes in eGFP-gephyrin clustering in the presence of V5-CB1 and CB2 splice isoforms. (A-E'') 8+7 DIV neurons co-transfected with either V5-CB1$_{SH3+}$, V5-CB1$_{SH3-}$, V5-CB2$_{SH3+}$ or V5-CB2$_{SH3-}$ isoform and eGFP-gephyrin (green), stained for the V5-tag (blue) and for presynaptic marker VGAT/VGLUT (magenta). Alterations in the morphology of eGFP-gephyrin synaptic clusters was observed (Scale bar 10µm). **(F-K'')** 8+7 DIV neurons were co-transfected with either V5-CB1$_{SH3+}$, V5-CB1$_{SH3-}$, V5-CB2$_{SH3+}$ or V5-CB2$_{SH3-}$ isoform (blue) and eGFP-gephyrin (green) and stained for GABA$_A$R γ2 subunit (magenta). The localization of eGFP-gephyrin clusters with γ2 subunit was observed (Scale bar 10µm). **(L)** Histogram of eGFP-gephyrin synaptic cluster density per 20 µm dendrite (DIV 8+7) shows a shift to the right in the presence of all four V5-CB isoforms. **(M)** Cumulative probability distribution of eGFP-gephyrin synaptic cluster size shows that CB2 isoforms specifically enhance gephyrin cluster size. Statistical analysis for cluster density; One-way ANOVA, Bonferroni post-hoc test, p=0.85; size analysis: Kruskal-Wallis non parametric test, Dunn's multiple comparison test p<0.0001.

Analysis of eGFP-gephyrin clusters in neurons transfected with $CB2_{SH3+}$ and $CB2_{SH3-}$ showed a significant increase in cluster size (0.38±0.01 μm^2 versus 0.54±0.02 μm^2 and 0.56±0.02 μm^2, respectively) (Kruskal-Wallis test; $p<0.0001$), but this was not the case in neurons co-expressing $CB1_{SH3+}$ or $CB1_{SH3-}$ (Fig. 6M). This result suggests that the longer protein half-life of the CB2 isoforms (Fig. 4A-D) might contribute towards the observed gephyrin cluster enlargement.

Collybistin C'-terminal sequence is essential for its function.

If CB turnover is an essential determinant for gephyrin cluster dynamics at GABAergic synapses; enhancing CB protein stability should stabilize eGFP-gephyrin clusters. In order to clarify this, we co-transfected DIV 8 neurons and analyzed for changes in eGFP-gephyrin density and size at DIV 8+7. Analysis of neurons co-transfected with V5-$CB1_{SH3+}$(K491R/K492R) or V5-$CB1_{SH3-}$(K431R/K432R) mutants and eGFP-gephyrin, comparing them to neurons transfected with V5-$CB1_{SH3+}$, V5-$CB1_{SH3-}$ or eGFP-gephyrin alone, showed significant increase in eGFP-gephyrin clustering (Fig. 7A-C'). Quantification of changes in synaptic cluster density confirmed the significant difference between V5-$CB1_{SH3+}$ and V5-$CB1_{SH3+}$(K491R/K492R) (7.69±0.6 versus 11±0.9; one-way ANOVA, $p<0.0001$) as well as V5-$CB1_{SH3-}$ and V5-$CB1_{SH3-}$ (K431R/K432R) (6.4±0.7 versus 10.89±0.8; one-way ANOVA, $p<0.0001$) (Fig. 7D). However, there was no significant difference in eGFP-gephyrin cluster size (Suppl. Fig. 3A-B). Our data suggested that in addition to protein stability, C'-terminal sequence also contributes towards CB function and regulation of eGFP-cluster size and density. In order to test the influence of CB C'-terminal sequence for eGFP-gephyrin cluster density and size regulation, we used the C'-terminal deletion mutants V5-$CB\Delta C_{SH3+}$ and V5-$CB\Delta C_{SH3-}$ (Suppl. Fig. 1B), co-transfected them with eGFP-gephyrin into DIV 8 hippocampal neurons, and analyzed changes in gephyrin cluster size and density at DIV 8+7 (Fig. 7E-F).

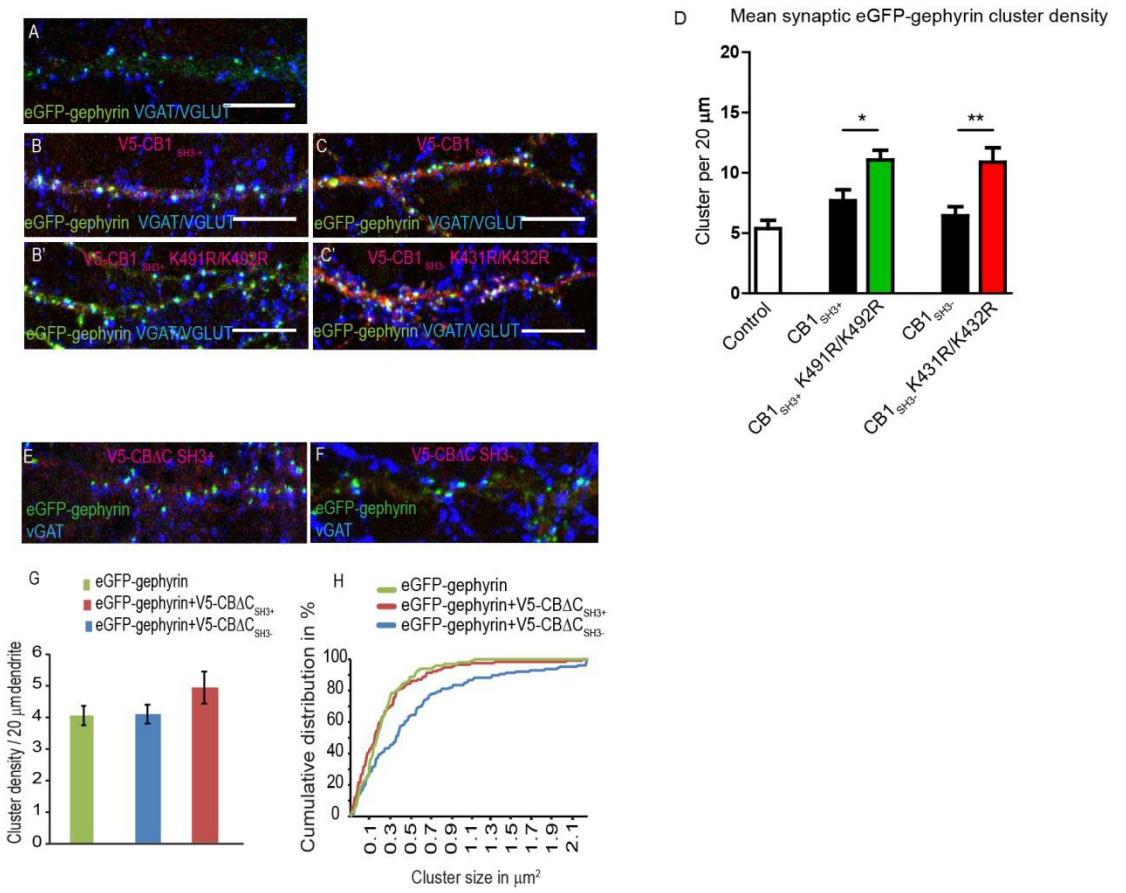
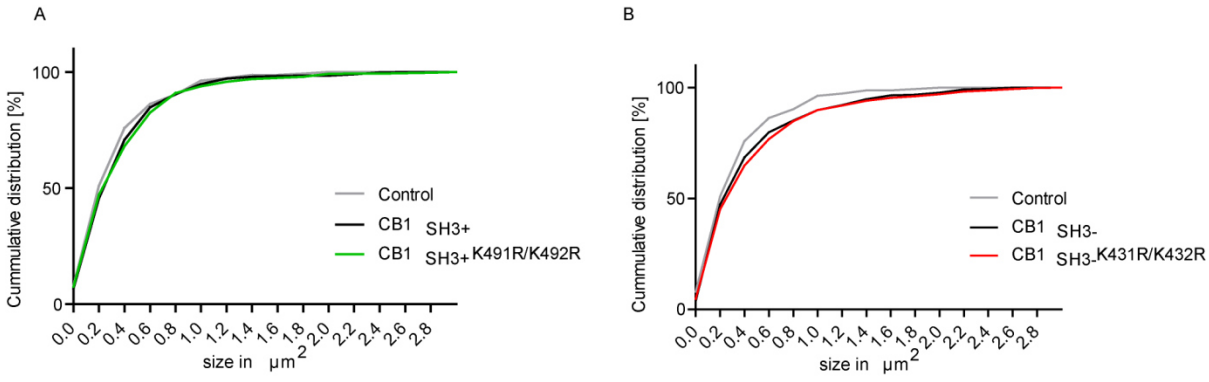


Figure 7: Morphological changes to eGFP-gephyrin clustering after CB stabilization. (A-C') Morphological analysis of neurons co-transfected with eGFP-gephyin (green) and CB1 ubiquitin mutants $CB1_{SH3+}$ K491R/K492R or $CB1_{SH3+}$ K491R/K492R stained the V5-tag (red) and for presynaptic marker VGAT/VGLUT (blue). **(D)** Quantification of eGFP-gephyrin synaptic cluster density in neurons co-transfected with $CB1_{SH3+}$ K491R/K492R or $CB1_{SH3-}$ K431R/K432R mutants show enhanced eGFP-gephyrin cluster density (DIV 8+7). **(E-F)** Morphology of eGFP-gephyrin clusters in neurons co-transfected with V5-CBΔC_{SH3+} or V5-CBΔC_{SH3-}. **(G)** Quantification of synaptic eGFP-gephyrin cluster density in neurons co-transfected with V5-CBΔC_{SH3+} or V5-CBΔC_{SH3-}. **(H)** Quantification of synaptic eGFP-gephyrin cluster size in neurons co-transfected with V5-CBΔC_{SH3+} or V5-CBΔC_{SH3-}. One-way ANOVA with Kruskal-Wallis non parametric test, Dunn's multiple comparison test $p<0.0001$).

We could not observe any significant differences in eGFP-gephyrin cluster density between control, V5-CBΔC_{SH3+}, and V5-CBΔC_{SH3-} transfected neurons (4±1.4 versus 4±0.9 and 5±1.5; one-way ANOVA, Kruskal-Wallis post-hoc analysis $p=0.7$) (Fig. 7G). However, we observed a significant increase in eGFP-gephyrin cluster size in neurons co-transfected with V5-CBΔC_{SH3-} (0.29±0.01 µm² versus 0.59±0.05 µm²; one-way ANOVA, Kruskal-Wallis post-hoc analysis $p<0.0001$) (Fig. 7H). The prevailing view envisions a specific role for the SH3 domain in regulating CB

structure and function (Reddy-Alla et al., 2010). Our data, however, implicate both the SH3 domain and the C'-terminus for isoform-specific CB conformation and function.



Suppl. Figure 3: eGFP-gephyrin synaptic cluster size in neurons co-transfected with V5-CBΔ_{SH3+}, V5-CBΔ_{SH3-} or their Ub mutants. (A) Cumulative probability distribution of eGFP-gephyrin cluster size in neurons co-transfected with V5-CB1$_{SH3+}$ or V5-CB1$_{SH3+}$K491R/K492R compared to control (DIV 8+7). (B) Cumulative probability distribution of eGFP-gephyrin cluster size in neurons co-transfected with V5-CB1$_{SH3-}$ or V5-CB1$_{SH3-}$K421R/K422R. One-way ANOVA, Kruskal-Wallis test; $p<0.5$.

CB isoforms in primary rat neurons show dendritic mRNA localization.

Identification of activity-dependent CB isoform mRNA splicing and characterization of isoform- specific differences in protein turnover implicated local CB synthesis for dynamic adaptations to neuronal activity. Hence, we examined the subcellular localization of CB mRNAs in cultured hippocampal rat neurons using QuantiGene ViewRNA™ technique (Panomics), which allows visualization at single transcript resolution using FISH (Will et al., 2013). The CB1 transcript harbors a unique 30 amino acid C'-terminus sequence, while CB2 C'-terminus is shorter (unique 8 amino acids) (Suppl. Fig. 1A); hence, we could only design probes specific to CB1 and compared this isoform against a PanCB probe. Consistent with our observations *in vivo,* we could detect both CB1 and PanCB transcripts at both DIV 5 and DIV 18. *In situ* hybridization signals were distributed over neuronal somata and dendrites, as illustrated for CB1 and PanCB mRNAs at DIV 18 (Fig. 8A-B). Although the two probes showed a distinct overlap in the neuronal soma, we saw very little overlap in the dendrites (Fig. 8A), suggesting that CB isoforms are transported and localized at distinct sites along the dendrites. Given that the PanCB probe should also recognize

the CB1 probe, and hence we should see a complete co-localization for CB1, we often found partial co-localization possibly due to steric hindrance. The localization of the CB mRNA isoforms in the neuronal dendrite is further demonstrated with an overlay of the differential interference contrast (DIC) image (Fig. 8B). We confirmed the specificity of our probes by down-regulating endogenous CB expression using specific shRNA (Fig. 8C).

A wide range of gephyrin cluster size and density is observed *in vivo*, suggesting that stable CB isoforms might be associated with stable GABAergic synapses, facilitating formation of larger gephyrin clusters; whereas more dynamic CB isoforms could be associated with smaller gephyrin clusters localized at transient synapses. In order to test this idea, we combined the QuantiGene ViewRNA™ technique with our neuronal transfection protocol to see whether eGFP-gephyrin co-localized with dendritic CB mRNA. Furthermore, we also wanted to see if we could morphologically associate eGFP-gephyrin cluster size with CB1 or PanCB transcript localization. Interestingly, we could co-localize some of the eGFP-gephyrin clusters with CB1 and PanCB probes (Fig. 8D; top panel). We could distinctly associate smaller eGFP-gephyrin clusters with CB1 probe (Fig. 8D; white arrowhead) and larger eGFP-gephyrin cluster with PanCB probe (Fig. 8D; white arrow), showing CB mRNA-ased eGFP-gephyrin cluster size distribution. These observations suggested that both mRNA and protein regulation might contribute to functional distinctions between the isoforms.

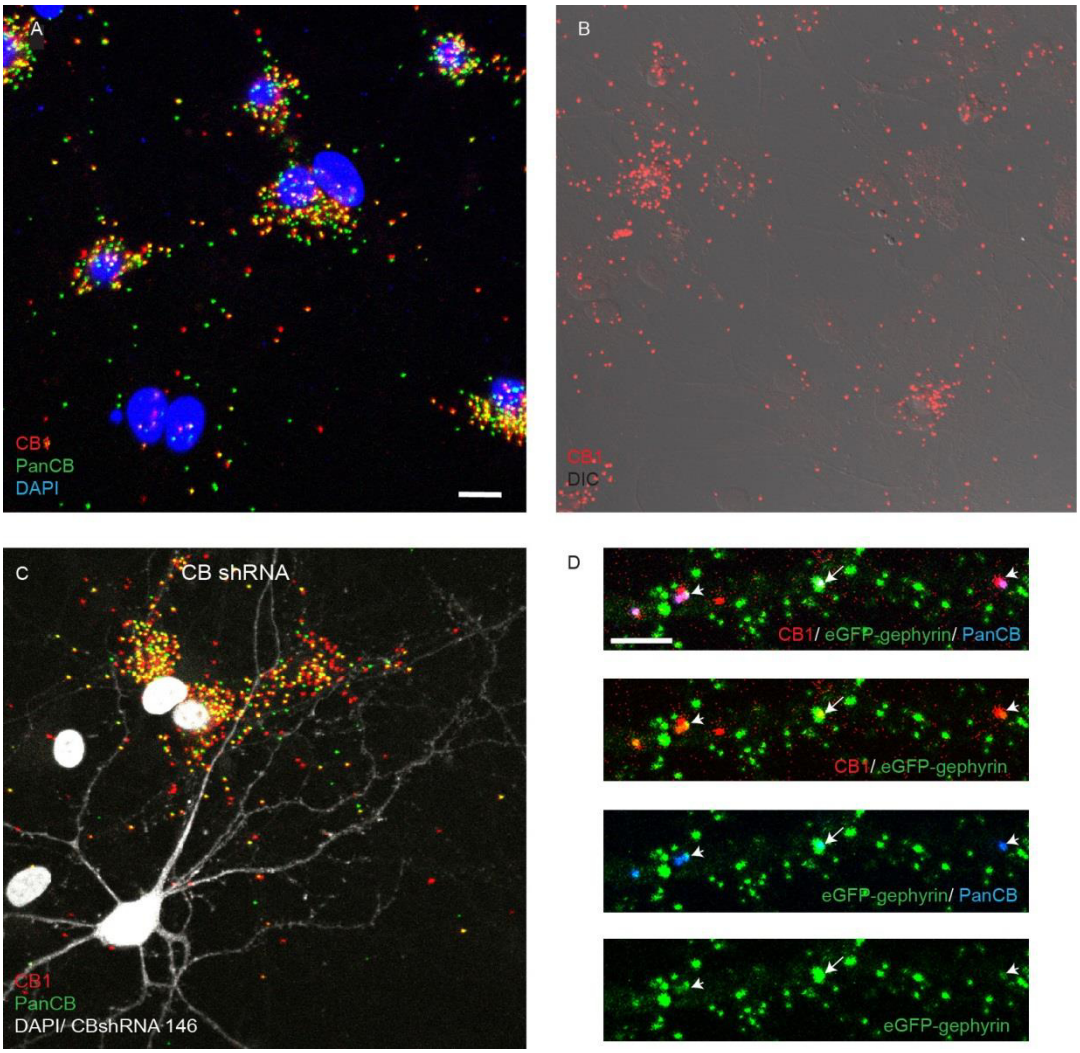


Figure 8: FISH analysis of CB transcripts in rat hippocampus culture. (A) We designed probes specific for CB1(red) and PanCB (green) to detect their expression and localization in cultured neurons using Quantigene (Affymetrix). **(B)** DIC image of the hippocampal neurons to demonstrate the presence of CB transcripts in the neuronal dendrites. **(C)** shRNA against endogenous CB shows the specificity of our probe design. DAPI and shRNA are depicted in white. (D) FISH analysis in neurons transfected with eGFP-gephyrin to look for co-localization of CB1 and PanCB probes with eGFP-gephyrin clusters. Smaller eGFP-gephyrin clusters are co-localized with CB1 positive probes (white arrowhead), while larger eGFP-gephyrin clusters are associated with PanCB probe (white arrow).

DISCUSSION

In this study we provide evidence that CB is an essential regulator of gephyrin clustering, which is able to substitute for the lack of postsynaptic receptors in neurons from γ2-KO mice. Furthermore, we demonstrate that CB isoforms are differentially regulated at both mRNA and protein level, likely endowing them with distinct, synapse-specific roles within neurons. Interestingly, differential regulation of CB isoform transcripts is not due to a transcriptional effect, but rather a post-transcriptional splicing mechanism, specifically regulated by either CaMKII or CaMKIV signaling. Our data also demonstrate localization of CB transcripts in dendrites, opening up the possibility for neuronal activity-dependent local protein synthesis, possibly contributing to GABAergic synaptic plasticity, similar to the role of dendritic protein synthesis at excitatory synapses for facilitation of long-term potentiation (LTP) (Liu-Yesucevitz et al., 2011). At the protein level, using heterologous expression, we reveal regulatory differences by showing that CB2 isoforms are significantly more stable compared to the CB1 isoforms. Furthermore, by identifying specific lysine residues within CB C'-terminus that determine isoform-specific stability, our data emphasizes the importance of an unique C'-terminus sequence for regulating overall protein conformation and stability. Taken together, we present a model in which CB1 isoforms undergo local translation and fast turnover to regulate activity-dependent adaptations of postsynaptic gephyrin clusters; whereas CB2 isoforms, on the other hand, bind to and stabilize gephyrin in the soma for transport to GABAergic synapses that are less dynamic and more stable (Fig. 9).

CB isoform-specific regulation of GABAergic synapse remodeling

Overexpression studies of various CB isoforms in neurons have failed to provide a functional understanding of gephyrin cluster regulation. We present data showing fundamental differences in mRNA and protein regulation that distinguish CB isoforms. In particular, we did not uncover any development dependent splicing differences between CB isoforms (unpublished); however, neuronal activity under CaMKII and/or CaMKIV signaling selectively affects the expression of CB1 and CB3, but not CB2, revealing that CB2 is unlikely to contribute to activity-dependent remodeling of gephyrin postsynaptic clusters. Overall, neuronal pre-mRNA splicing

regulation is emerging as an important determinant of functional diversity in different neuronal subtypes for dynamic synapse regulation. Neuronal activity-facilitated CaMKIV signaling was shown to regulate splicing factor SAM68 function for neurexin splicing in neurons (Iijima et al., 2011). More recently cell type-specific expression of splicing factors SLM1 and SLM2 was shown to shape the molecular repertoire of synaptic adhesion molecules by regulating the availability of neurexin-1, -2 and -3 (Iijima et al., 2014). Hence, unmasking of mRNA splicing differences between CB isoforms offers the essential first step towards delineating neuronal subtypes and/or synapse specific isoform function.

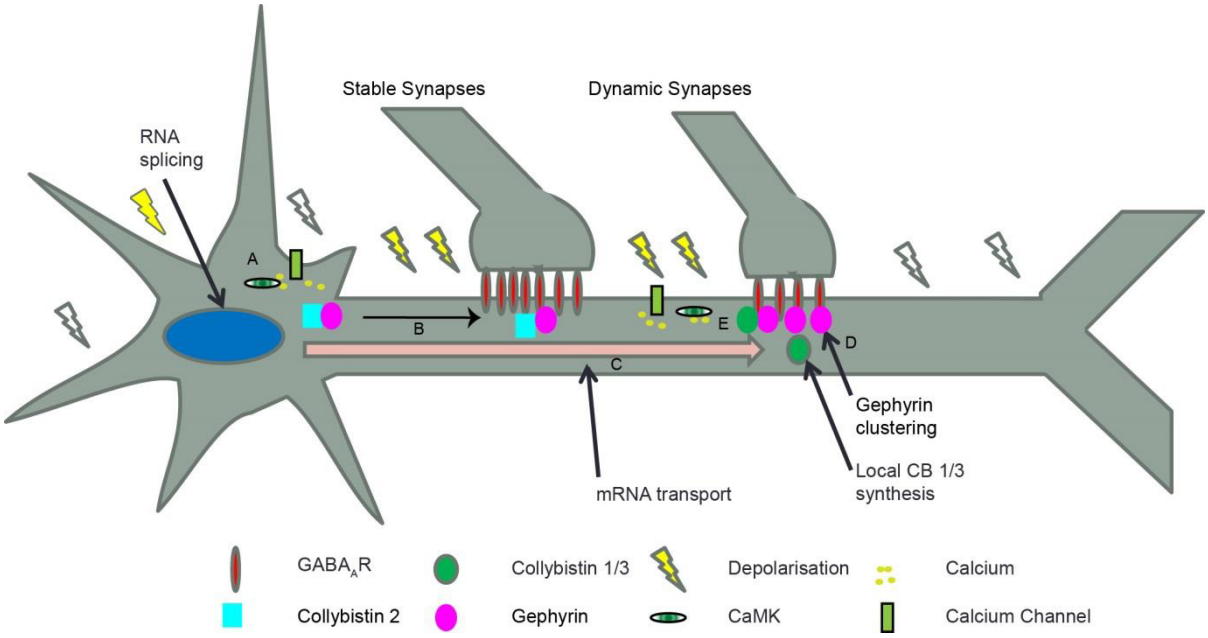


Figure 9: Model explaining the possible role of CB isoforms for plasticity changes at GABAergic synapses. (A) Activity-dependent and CaMK-dependent splicing of CB mRNA isoforms. **(B)** CB2 somatic translation stabilizes gephyrin and transports it to stable GABAergic synapses. **(C)** Dendritic transport of CB1 and CB3 mRNAs after activity dependent splicing changes for dendritic translation. **(D)** Local protein synthesis of CB1 and CB3 splice isoforms promote gephyrin clustering at dynamic GABAergic synapses. **(E)** Activity-dependent and Ca^{2+} dependent mechanisms facilitate Ub mediated CB1/CB3 protein turnover at dynamic synapses to promote gephyrin cluster loss and $GABA_AR$ internalization.

Differential protein stability between CB isoforms

We further identified differences in protein regulation between CB isoforms. CB1 isoforms appear to have a faster protein turn over compared to their CB2 counterparts suggesting CB1-containing synapses could exhibit dynamic gephyrin clustering (Fig. 9). By generating and characterizing V5-CBΔC_{SH3+} and V5-CBΔC_{SH3-} mutants, we demonstrate that, in addition to the SH3 domain, the C'-terminus also plays an important role in the regulation of gephyrin clustering. Involvement of the ubiquitination proteasome pathway (UPP) in regulating CB turnover is not surprising, as this pathway regulates many receptors and scaffolding proteins (Lin and Man, 2013). A link between UPP and synaptic plasticity has been demonstrated by bilateral infusion of the proteasome inhibitor lactacystin in the CA1 area to induce retrograde amnesia in rodents (Lopez-Salon et al., 2001). The necessary components of the UPP have been identified at synapses and postsynaptic density further linking synaptic plasticity and local protein homeostasis (Cajigas et al., 2010). Our analysis of $CB2_{SH3+}$ and $CB2_{SH3-}$ lysine mutants offer specific insights into isoform-specific regulation of CB isoforms by the UPP. For example K468 ($CB2_{SH3+}$) and K408 ($CB2_{SH3-}$) are very likely modified by another post-translational modification, such as acetylation or SUMOylation, which in turn might affect the ubiquitination status of K439 ($CB2_{SH3+}$) and K372 ($CB2_{SH3-}$). Such cross-talk between different post-translational modifications on either different or the same residues within a molecule is not unusual; we have recently shown that gephyrin is also a substrate for phosphorylation, acetylation and SUMOylation (Tyagarajan and Fritschy, 2014). Intra- and inter-molecular interactions between the NLGN family of trans-synaptic molecules has been described in literature (Poulopoulos et al., 2012). Hence, our data showing inter- and intra-molecular interactions between CB isoforms could allow for multiple interaction sites within a single GABAergic synapse and facilitate maintenance of the GABAergic postsynaptic apparatus.

Dendritic localization of CB mRNA

More than 2500 species of mRNAs have been identified in the dendrites and axons of neurons, suggesting that key regulation of synaptic signaling and dendritic excitability have a local rather than somatic source (Cajigas et al., 2012). In line with these observations, our data localize CB transcripts in dendrites, demonstrating the

importance of local protein synthesis and synaptic protein turnover for inhibitory synapse plasticity. NMDA activation-dependent translocation of CaMKIIα from spine synapses (sites for excitatory inputs) to shaft synapses (sites for inhibitory inputs) has been reported earlier (Marsden et al., 2010). Identification of gephyrin clusters in spines (Chen et al., 2012; van Versendaal et al., 2012) also shows that the spatial segregation between excitatory and inhibitory synapses has exceptions to the rule. Given that activity-dependent mRNA splicing of CB1 and CB3 isoforms is under specific control of CaMK signaling, and CaMKIIα signaling at spines for LTP and other forms of synaptic plasticity is well characterized, one can envisiage, for instance, CB1/CB3 local protein synthesis and localization at spine GABAergic synapses, while CB2 is localized to shaft GABAergic synapses. Such spatial distribution of CB isoforms would also explain why some of the gephyrin clusters are more dynamic than others (See Model in Fig. 9).

While disruption in GABAergic transmission and/or synaptic structure organization has been associated with many neuropsychiatric and neurodevelopmental disorders (Tyagarajan and Fritschy, 2014), the underlying mechanism(s) for the observed functional defects remain(s) largely unclear. Identification and characterization of specific regulatory events that are upstream of gephyrin scaffold formation and GABAergic synapse function offers the essential first step towards understanding the mechanisms underlying such complex disorders.

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IN VIVO STUDY: COLLYBISTIN-DEPENDENT MATURATION AND SYNAPTIC INTEGRATION OF ADULT BORN NEURONS IN THE HIPPOCAMPUS

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In preparation

My contribution to this study was the generation of all constructs and production of the virus, all experiments and analysis of the overexpression of $CB1_{SH3-}$ in α2-KO mice and the silencing of CB, as well as in the writing process.

ABSTRACT

Collybistin (CB) is a neuron-specific guanine nucleotide exchange factor (GEF), activating the small Rho-GTPases Cdc42 and TC10. CB was discovered as an interactor of gephyrin, the main scaffolding protein of GABAergic and glycinergic synapses, and is essential for proper postsynaptic clustering of gephyrin and $GABA_A$ receptors in many parts of the CNS. The precise function(s) of CB are not understood, in particular the significance of Cdc42 activation and the role of distinct CB isoforms (CB1-CB3) that are generated by alternative mRNA splicing. The aim of this study was to investigate *in vivo* the effects of CB overexpression or shRNA-mediated silencing on GABAergic synapse formation and morphological development of adult-born neurons in the dentate gyrus, which are derived from a pool of actively dividing neural precursor cells. To this end, we injected into the dorsal hippocampus of adult mice retroviral vectors encoding eGFP (control), eGFP-$CB1_{SH3-}$ (lacking the SH3 domain), or eGFP and a shRNA construct targeting all CB isoforms, and examined the morphological development of transfected newborn granule cells between 14 and 42 days post-injection (dpi). Overexpression of eGFP-$CB1_{SH3-}$ was also done in *Gabra2*-null mice, which have a strong deficit in postsynaptic gephyrin clustering in hippocampal neurons. In wild-type mice, both the overexpression of eGFP-$CB1_{SH3-}$ and shRNA-mediated CB silencing impaired the migration of adult-born granule cells into the granule cell layer and led to dendritic hypertrophy. CB silencing also led to the formation of supernumerary immature spines in fully developed cells (42 dpi). Unexpectedly, CB overexpression or silencing had no major effect on the formation of postsynaptic gephyrin or $GABA_A$ receptor $\alpha1$ or $\alpha2$ subunit clusters unlike that observed *in vitro* or in CB-knockout mice. In *Gabra2*-null mice, CB overexpression restored gephyrin clustering and even caused a transient compensatory increase in the density of $\alpha1$ subunit clusters. All these effects are indicative of constitutive activation of small Rho-GTPases, notably Cdc42, induced either upon overexpression of eGFP-$CB1_{SH3-}$ or CB-silencing. Therefore, our results suggest that under baseline conditions, CB largely prevents activation of Cdc42, possibly by sequestering it away from other Rho-GEFs. This inhibition is relieved upon CB-silencing, or can be reverted to increased Cdc42 activation upon eGFP-$CB1_{SH3-}$ overexpression. Since the GEF activity of CB depends on its molecular conformation and on binding to membrane phospholipids, we propose that overexpression of eGFP-$CB1_{SH3-}$ tips the balance towards increasing the pool of conformationally active CB at GABAergic synapses, notably to rescue gephyrin clustering in *Gabra2*-null mice.

INTRODUCTION

In vertebrate CNS, GABA is the main inhibitory neurotransmitter, acting primarily by activating $GABA_A$ receptors ($GABA_ARs$), which form Cl^--permeable ligand-gated channels assembled from a family of 19 subunit genes (Olsen and Sieghart, 2008). $GABA_ARs$ are concentrated at postsynaptic sites by interacting with a protein scaffold organized by gephyrin, a multifunctional protein initially isolated with purified glycine-receptor preparations (Pfeiffer et al., 1982). Gephyrin forms a regular lattice by auto-oligomerization (Sander et al., 2013), anchoring receptors and other signaling molecules of the postsynaptic density to the cytoskeleton (Kneussel and Betz, 2000). There is increasing evidence that regulation of gephyrin postsynaptic clustering properties is a major determinant of GABAergic synapse formation, function, and plasticity (reviewed in (Tyagarajan and Fritschy, 2014)), and that multiple proteins and signaling cascades are involved in this process.

Collybistin (CB) is a brain specific guanine nucleotide exchange factor (GEF) of the dbl-family, discovered in a two-hybrid of gephyrin binding partners (Kins et al., 2000). In recombinant systems, CB facilitates membrane targeting of gephyrin, and in neurons, it increases the number and strength of GABAergic synapses (Harvey et al., 2004; Chiou et al., 2011; Tyagarajan et al., 2011a; Körber et al., 2012). In rodents, *Arghef9*, the gene encoding CB, is alternatively spliced into three isoforms (CB1-CB3), differing in their C'-terminus; CB3 is identical to the human analogue hPEM2. Structurally, CB harbors a dbl-homologue (DH) and a pleckstrin homologue (PH) tandem domain. This tandem domain is conserved in all dbl-family RhoGEFs. In addition, CB has a scr-homology 3 domain (SH3), which can be spliced out in each of the three isoforms. As a RhoGEF, CB selectively activates the small Rho-like GTPases Cdc42 and TC10 through its DH domain (Xiang et al., 2006; Mayer et al., 2013), but the intrinsic GEF activity is very low and the relevance of the activation of Cdc42 or TC10 through CB *in vivo* is still unclear (Jaiswal et al., 2013).

In vivo, the importance of CB at GABAergic synapses was revealed in CB-KO mice, which exhibit a region- and cell-specific loss of postsynaptic gephyrin and $GABA_AR$ clusters, along with altered synaptic plasticity, increased anxiety, and impaired spatial learning (Papadopoulos et al., 2007; Papadopoulos et al., 2008). Therefore, to elucidate the mechanisms underlying this complex phenotype, the role of individual

CB domains, shared among CB1-3, has been investigated intensively. The PH domain interacts with phosphatidylinositol-3-phosphate (PI3P) and is essential for membrane anchoring of CB (Kalscheuer et al., 2009; Reddy-Alla et al., 2010; Tyagarajan et al., 2011a); and, therefore, for proper postsynaptic gephyrin clustering. The DH domain binds Cdc42 and gephyrin, and structural evidence suggests mutual exclusion, rising questions how (and when) CB activates Cdc42. Further, silencing GEF activity by point-mutagenesis does not impair gephyrin clustering (Reddy-Alla et al., 2010). The role of the SH3 domain also is elusive. In analogy with another Rho-GEF (Murayama et al., 2007), it has been proposed to control the accessibility of the DH and PH domains (and thereby CB's functions) in a conformation-dependent manner. In particular, this mechanism was proposed to regulate the formation of GABAergic synapses upon interaction between CB and the cell-adhesion molecule neuroligin2, as well as the $GABA_AR$ $\alpha2$ and $\alpha3$ subunits (Poulopoulos et al., 2009; Saiepour et al., 2010). This conformational change in CB was also shown to enhance the affinity of the PH domain to PI3P in the plasma membrane, favoring gephyrin clustering (Soykan et al., 2014). However, upon CB overexpression in cultured neurons, the presence/absence of the SH3 domain had little influence on gephyrin clustering and GABAergic synapse formation (Chiou et al., 2011; Tyagarajan et al., 2011a). Further, the role of Cdc42 down-stream of CB remains fully enigmatic. Targeted deletion of Cdc42 was reported not to affect gephyrin clustering (Reddy-Alla et al., 2010), whereas, *in vitro*, Cdc42 overexpression can rescue gephyrin clustering impaired by the presence of a CB mutant construct lacking the PH domain. In addition, Cdc42, either alone or in combination with CB, modulates the size and shape of postsynaptic gephyrin clusters (Tyagarajan et al., 2011a). Cdc42 interacts with both CB and gephyrin, and the three proteins can form a complex, which might represent a functional unit in neurons. Finally, we have shown that the C'-terminal domain, which differentiates CB isoforms, regulates their half-life in an ubiquitin-dependent manner (de Groot et al., submitted). Collectively, these findings suggest distinct roles for CB1 and CB2 based on their availability and subcellular distribution within neurons. However, deletion of the C'-domain does not abolish the major functions of CB (de Groot et al., submitted), and it has not yet been determined whether CB1-3 have different regional or temporal expression pattern in CNS (Patrizi et al., 2012).

During CNS development, GABAergic transmission regulates key steps of neurogenesis and neuronal circuit formation (Behar et al., 2000; Represa and Ben-Ari, 2005). A similar role has been observed for the regulation of adult neurogenesis (Bordey, 2007; Ge et al., 2007; Panzanelli et al., 2009), best seen upon targeted deletion of specific $GABA_AR$ subunits, which impairs cell migration, dendrite formation and synaptic integration (Duveau et al., 2011; Pallotto et al., 2012). An indirect approach to investigate the function(s) of CB in relation to the GABAergic system is to manipulate its expression in adult-born neurons and determine the effects on GABAergic synapse formation and neuronal maturation. Thus, in the present study, we used adult neurogenesis in the subgranular zone (SGZ) – giving rise to new dentate gyrus granule cells (Kempermann et al., 2004) – as an *in vivo* model system to further investigate the significance of CB heterogeneity.

We used retroviral vectors encoding either an eGFP-tagged CB isoform lacking the SH3 domain (eGFP-$CB1_{SH3-}$) or eGFP and CB shRNA, injected stereotaxically into the hilus of the dentate gyrus of adult mice. Expression of eGFP alone or scrambled shRNA was used as control. Differentiation and maturation of newborn virus-transduced granule cells were monitored between 14 and 42 days post-injection (dpi), using morphological techniques. $CB1_{SH3-}$ overexpression was tested in C57Bl6/J wild-type mice and *Gabra2*-deficient mice, to test whether it can rescue the impaired gephyrin clustering and neuronal plasticity induced by the removal of α2-$GABA_ARs$ (Duveau et al., 2011; Pallotto et al., 2012). A pan-CB shRNA, targeting a sequence found in all CB isoforms, was selected to effectively down-regulate CB and was tested in wild-type mice only. Collectively, the results unravel that CB exerts distinct effects in differentiating neurons, which might in part be mediated by interaction with gephyrin and in part upon regulation of Cdc42 (and possibly TC10) activity.

MATERIAL AND METHODS

Animals

All animal procedures were approved by the cantonal veterinary office of Zurich and performed in accordance with the European Community Council Directive 2010/63EU of 22 September 2010. During breeding and experiment the mice were housed in groups of 3-5 animals per cage under a 12 h light/dark cycle, with food and water provided *ad libitum*. α2-KO mice were generated at the Institute of Pharmacology and Toxicology of the University of Zurich on a C57BL/6J background and were described earlier (Panzanelli et al., 2011). The α2-KO allele was backcrossed for more than nine generations and maintained on a heterozygote background. Mice were genotyped by PCR analysis of ear biopsies. Experimental wild-type mice (C57Bl6/J) were bred at the animal facility of the Institute or directly ordered from Harlan Laboratories (The Netherlands) and habituated to the breeding facility for at least two weeks.

Plasmids

A non-replicative retroviral vector based on the Moloney murin leukemia virus was used as a backbone, containing either an enhanced green-fluorescent protein (eGFP) alone under the CAG promoter (pCAG-V-PRE-eGFP). pCMV-gp expressing *gag/pol* genes for packaging and pCMV-vsv-g used as envelop protein plasmids were a kind gift of Dr. Sebastian Jessberger (University of Zurich). The eGFP-CB1$_{SH3-}$ was cut out from the previously described pEGFP-C2-CB1$_{SH3-}$ vector and cloned into the pCAG-V-PRE-eGFP vector using BamHI and NotI as restriction sites. shRNA against *ArhGEF9* were ordered from OriGene Technologies (Rockville, USA) in a HuSH pRFP-C-RS plasmids (TF517554) and were designed against multiple splice variants at this gene locus with the following sequences; CTGGAGATTCCATCGTTAGTGCTGAGGCA targeting a conserved sequence of the CB SH3 domain, CTGATGAAGGACAGCCGCTATCAACACTT targeting all CB splice isoforms across species, CCTGCTTCTTAGAGCATCAAGATGGATTC and GAACCGAAACGCAGCCAGTCACCATTCTG targeting CB only in mouse. As a control a 29-mer scrambled shRNA cassette in pRFP-C-RS Vector (TR30015) was used. pCAG-V-PRE-eGFP panCB shRNA and pCAG-V-PRE-eGFP scramble shRNA was cloned from the original plasmids from OriGene by restriction digest using PstI, which was

introduced through PCR amplification before the U6 promoter and after the termination sequence. Then, the eGFP sequence and the shRNA cassette were packed into the retrovirus. pCR3-V5-CB1$_{SH3+}$ and pCR3-V5-CB1$_{SH3-}$ were described previously (de Groot et al., submitted).

Virus production

HEK-293T cells were cultured in DMEM containing 10% FCS and 1% penicillin-STrEPtomycin-glutamine. A DNA mix, containing 15 μg coding plasmid, 10 μg gp plasmid and 5 μg pCMV vsv-g plasmid in 100 μL 2.5 M $CaCl_2$ solution, was drop-wise added to a 2xHeBS buffer (0.283 M NaCl, 0.023 M HEPES acid, 1.5 M Na_2HPO_4, pH 7.05) under air ventilation. After 30 min incubation, the mix was added to the HEK-293T cultures. After 24 h the culture medium was changed with fresh media. Medium containing the virus was harvested 24 h and 48 h later, pooled and filtered through 0.2 μm pores. The filtrate was concentrated by two rounds of ultracentrifugation, the first for 90 min at 29`000 rpm at 4°C, re-suspended in cold PBS and the second with a 20% sucrose cushion for 120 min at 20`000 rpm at 4°C. The final pellet was re-suspended in cold PBS and stored in 10 μL aliquots at -80°C. The virus had a titer of approximately 10^8 cfu/mL, determined by serial dilution of the virus and its ability to infect HEK-293T cells.

Cell culture

Primary hippocampal neuron cultures were prepared as described previously (Buerli et al., 2007). Hippocampal cultures were transfected with pCAG-V-PRE-eGFP panCB shRNA and pCAG-V-PRE-eGFP scramble shRNA with 0.3 μg of total plasmid, using a combination of Lipofectamine 2000 (Life Technologies) and CombiMag (OZ Biosciences). The neurons were grown in 2 mL of growth media (Buerli et al., 2007) for 12 days prior to transfection. 1 mL of this conditioned media was transferred into a fresh 12-well dish prior to transfection. The plasmids were mixed in 30 μL of OptiMEM medium (Life Technologies), in a separate tube, master mix was prepared consisting of Lipofectamine 2000 (2 μL per sample) in OptiMEM (30 μL per sample). After incubating the Lipofectamine 2000 mix at room temperature for 5 min, 32 μL of this mix was added to the tube containing the DNA. CombiMag was diluted (1:10) in OptiMEM media and 2 mL of the diluted CombiMag was added to each of the samples and mixed thoroughly. The transfection mix was incubated at room

temperature for 15 min before adding to the neurons. The 12-well dish with the transfection reagents were placed over a magnetic plate inside the incubator. The transfection was stopped 25min later by transferring the coverslips into the fresh 12-well dish containing the conditioned media. The neurons were transfected after 12 days in vitro (DIV) and processed for immunofluorescence 7 days later.

HEK-293T cells were cultured at 37°C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). They were transfected with 1.5 μg DNA at 16 h post-plating using polyethylamine (PEI) according to the manufacturer's recommendation. The whole cell lysate was prepared 72 h post transfection using EBC buffer containing Complete-mini (Roche) and phosphatase inhibitor cocktail (Sigma).

Stereotactic intrahippocampal injections:

Adult male mice (8-12 weeks) weighing > 25 g were anesthetized with 5% isoflurane in oxygen during head fixation and trepanation; subsequently, isoflurane concentration was reduced to 1.5%. 1 μL of retrovirus encoding either eGFP, eGFP-$CB1_{SH3-}$, eGFP and panCB shRNA, or eGFP and scrambled shRNA was injected bilaterally into the hilus of the dentate gyrus, under stereotaxic guidance (coordinates from Bregma: antero-posterior -2 mm; lateral ±1.5 mm; dorso-ventral -2.3 mm from the skull). During the operation and recovery, mice were held on a warm pad, for recovery the mice were injected with 1 mg/kg buprenorphine (Temgesic, Essex Chemicals) and kept in a single cage until the behavior returned to normal.

Tissue preparation and immunohistochemistry

Perfusion

Mice deeply anesthetized with sodium pentobarbital (50 mg/kg; i.p) were perfused through the left ventricle with 20 mL ice-cold, oxygenated ACSF (125 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM glucose, pH7.4) at a flow rate of 10-15 mL/min. The brain was immediately extracted and a block containing the hippocampal formation fixed for 90 min in ice-cold paraformaldehyde solution (4% in 0.15 M sodium phosphate buffer, pH 7.4). Thereafter, the tissue was rinsed with phosphate-buffered saline (PBS) and stored

overnight at 4°C in 30% sucrose dissolved in PBS for cryoprotection (Notter et al., 2014).

Immunohistochemistry

The tissue was sectioned coronally from frozen tissue with a sliding microtome at a thickness of 50 µm and collected free-floating in PBS. Staining was performed under continuous agitation in primary antibody solution (Tris buffer (pH 7.4) containing 0.2 Triton X-100, 2% normal serum) for 72 h at 4°C (see Table 1 for the list of primary antibodies). Sections were then washed three times in PBS and incubated for 30 min at room temperature in the secondary antibodies coupled to a fluorochrome together with DAPI. They were washed again in PBS, mounted, air-dried and coverslipped with Dako fluorescence mounting medium. Tissue was kept in the dark at 4°C till they were analyzed.

Cultured neurons were fixed for 10 min in 4% PFA in 0.15 M sodium phosphate buffer, pH 7.4., rinsed in PBS and permeabilized with 0.01% Triton X-100 containing 10% normal goat serum. Immunohistochemistry was performed by incubating the cells with the primary antibodies diluted in PBS containing 10% normal goat serum for 60 min. After washing three times in PBS the cells were incubated with the secondary antibodies coupled to Cy3 or Cy5 (1:1000, Jackson ImmunoResearch) for 30 min, washed again three times in PBS, air-dried and mounted with fluorescent mounting medium (Dako Cytomation, Carpinteria, CA). The entire procedure was performed at room temperature.

Western blot analysis

After boiling the lysed HEK-293T cells in 5x SDS sample buffer containing 100 mM dithiothreitol (DTT) for 10 min at 72°C, they were loaded onto SDS-polyacrylamide gels and run at 140 V at room temperature. After transferring the protein bands onto PVDF membranes with constant 35 mA in Tris-glycine transfer buffer, WB were performed by blocking the membranes with 5% western blocking reagent (Roche Diagnostics) in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and later incubating with primary antibody overnight at 4°C. Secondary antibody coupled to IRDye® (LI-COR) was used to visualize the protein

bands. After WB the protein-band intensity was measured using LI-COR odyssey scanner and image studio.

Table 1

Target	Distributor	Description	Dilution
$GABA_AR$ α1 subunit	Fritschy lab	Guinea pig antiserum	1 : 5 000
$GABA_AR$ α2 subunit	Fritschy lab	Affinity purified guinea pig antiserum	1 : 1000
Gephyrin	Synaptic Systems, Göttingen, Germany	Mouse monoclonal, clone 7a, 147021	1 : 1000
GFP	Aves Labs Inc., Tigard, OR, USA	Chicken polyclonal, GFP-1020	1 : 2 000
VGAT	Synaptic Systems	Rabbit polyclonal, 131003	1 : 3 000
V5-tag	Acris, SanDiego, CA, USA	Mouse monoclonal SM1691	1: 3 000
Actin	Sigma-Aldrich, St. Louis, MO, USA	Rabbit polyclonal A2066	1: 10 000
panCB	ImmunoGenes AG, Zug, Switzerland	Rabbit, S515	1: 5 000

Data Analysis

For each group and time-point, at 3-4 mice were analyzed, representing a sample of 12 to 40 cells, depending on the analysis.

Microscopy and image processing

Immunofluorescence images synaptic proteins in sections containing labeled adult-born cells were acquired by laser scanning confocal microscopy 40 × lens, NA 1.4, 1024 × 1024 pixels (Zeiss LSM 700, Jena, Germany), taking stacks of images spaced by 0.4 μm spacing (pixel size, 0.9 μm). Images were analyzed using ImageJ (Wayne Rusband, National Institutes of Health, USA) applying background subtraction, a

Gaussian filter and intensity threshold segmentation. The density of puncta formed by pre- or postsynaptic markers was determined by co-localization analysis on each single image. Spine quantification was performed using the software NeuronStudio (CNIC, Mount Sinai School of Medicine, NY, USA).

Virus-transduced, eGFP-positive adult-born cells were visualized either by epifluorescence or confocal laser scanning microscopy. Images were acquired at high-resolution on a Zeiss AxioImager Apotome microscope (20x lens, NA 0.8) for analyzing cell migration and dendrite formation (Sholl analysis). For migration analysis, nuclei in the granule cell layer were counterstained with DAPI and the radial distance between eGFP-positive cell bodies and the base of the GCL was measured. Sholl analysis of the dendritic tree was performed using Fiji software (http://fiji.sc/Fiji) (Schindelin et al., 2012) using virtual concentric circles spaced by 10 μm and centered on the cell body. The area under the curve (AUC) was calculated from the resulting function (number of intersections as a function of distance from the soma) and used for statistical comparison.

Statistics

Statistical analyses were performed using Prism software (GraphPad, La Jolla, CA). Data are presented as mean± SEM. Comparison over multiple groups was done using one-way ANOVA, followed by Tukey *post-hoc* test or two-way ANOVA, followed by Bonferroni *post-hoc* test. Two-group comparisons were made with unpaired Mann-Whitney U-test and Kolmogorov-Smirnoff test was used for the cumulative probability distribution of the migration analysis. The threshold for rejecting the null hypothesis was set at $P < 0.05$.

RESULTS

Collybistin modulates the maturation of adult-born dentate gyrus granule cells

CB is a multi-functional protein regulating gephyrin postsynaptic clustering and the activity of the small Rho-GTPases Cdc42 and TC-10. Among the multiple CB splice variants, $CB1_{SH3-}$ has a very short half-life, suggesting the possibility of "on demand" modulation of specific processes, notably during phases of enhanced neuronal and synaptic plasticity (de Groot et al., submitted). To test this possibility, we over-expressed $CB1_{SH3-}$ in adult-born granule cells using retroviruses encoding either eGFP-$CB1_{SH3-}$ or eGFP as control and analyzed the effects on migration, morphology, and GABAergic synapse formation at 14, 28 or 42 dpi. These time-points represent three distinct phases of maturation of adult-born GCs and allow direct comparison with a previous study from our lab (Duveau et al., 2011).

GCs overexpressing $CB1_{SH3-}$ penetrated less deeply into the GCL, with 80-90% of cells remaining within < 20 μm from the SGZ border at each of the three time-points examined, whereas in the control group, >25% of GCs migrated more than 20 μm. The difference was significant at 28 and 42 dpi (Kolmogorov-Smirnov test; Fig. 1B-B''). This finding suggested that $CB1_{SH3-}$ negatively regulates cytoskeletal reorganization required for cell motility. However, all virally-transduced GCs had moved away from the SGZ, indicating that migration *per se* was not impaired.

Figure 1. $CB1_{SH3-}$ overexpression in wild type mice affects cell migration and dendritic arborizations. ***A,A'.*** Double immunofluorescence analysis of either α2 (red) or α1 (blue) subunit clusters on dendrites expressing eGFP-only (A) or eGFP $CB1_{SH3-}$ (A'); the framed areas are enlarged on the right to display the localization of the clusters (white) on eGFP-positive segments. Scale bar, 5 μm. ***B.*** Cumulative distribution analysis of the migration distance covered by virally-transduced GCs at (B) 14, (B') 28, and (B'') 42 dpi. A significant leftward shift was evident in the distribution of eGFP-$CB1_{SH3-}$ GCs compared to WT at the later time-points (Kolmogorov–Smirnov test, **P* < 0.05). ***C.*** Quantification of dendritic arborization by Sholl analysis at (C) 14, (C') 28 and (C'') 42 dpi of control (eGFP-only) and eGFP-$CB1_{SH3-}$ GCs. Comparison of the area-under-the-curve was significantly different between control and eGFP-$CB1_{SH3-}$ expressing cells at 28 and 42 dpi, indicating an increase in dendritic arborization (**P* < 0.05), in particular distally. ***D.*** Analysis of the total dendritic length showed a significant increase in GCs expressing eGFP-$CB1_{SH3-}$ at 28 and 42 dpi (mean ± SEM, ***P* < 0.01, Bonferroni *post-hoc* test), mirroring the increase in dendritic arborization. ***E - G.*** Quantification of the density of gephyrin, α2 subunit and α1 subunit clusters localized on GFP-positive dendrites, divided in proximal and distal segments at 14, 28 and 42 dpi. Proximal corresponds to the primary segment from the soma until the first bifurcation, and distal segments were located in the ML.

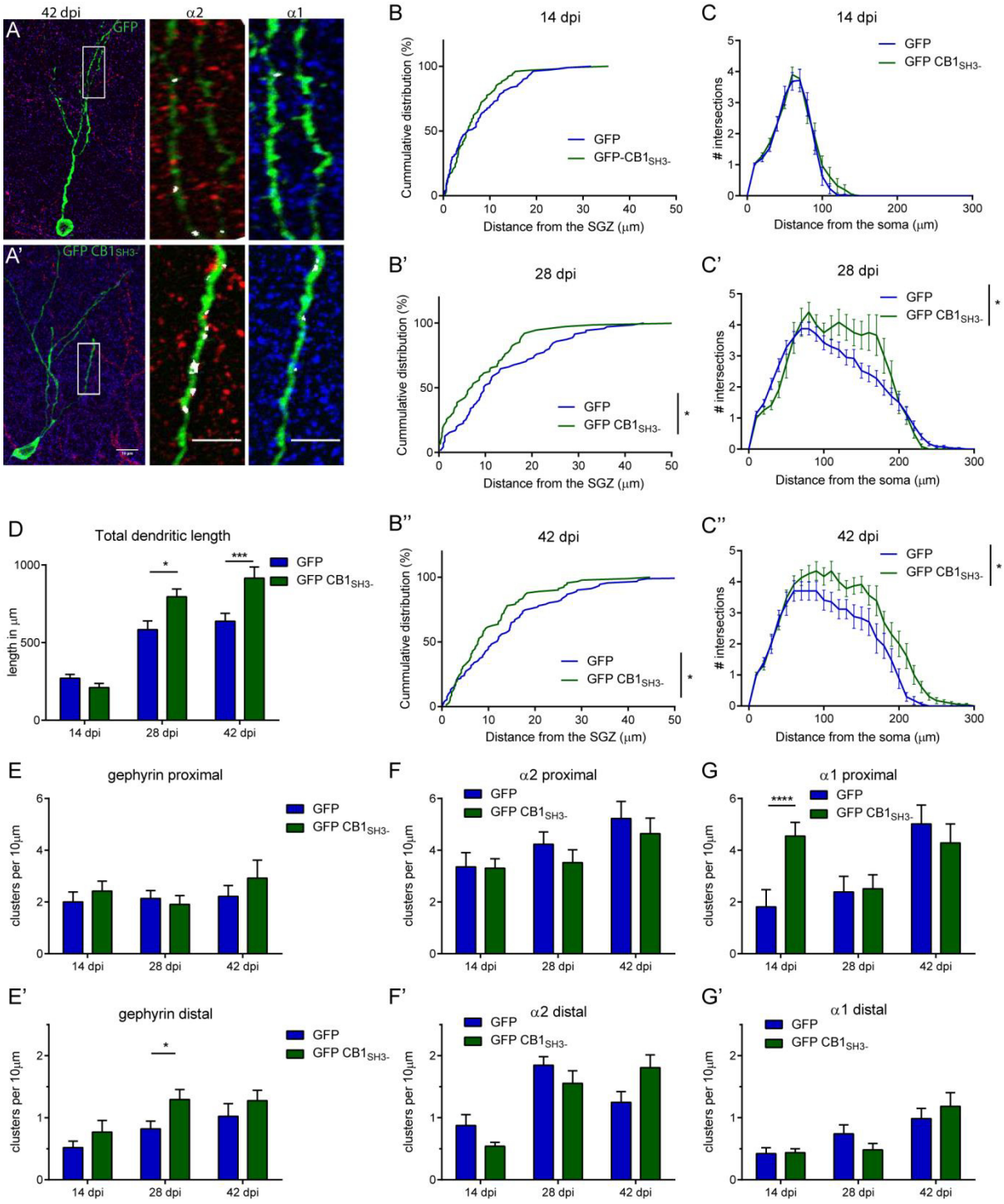


Figure 1 (legend on previous page)

(**cont. Figure 1**) No change in gephyrin cluster density was observed in the primary segment (E), and only a transient increase occurred at 28 dpi in distal segments (E') (mean ± SEM, **P* < 0.05, Bonferroni *post-hoc* test). Whereas α2 subunit cluster density was not affected by eGFP-CB1$_{SH3-}$ overexpression (F,F'), α1 subunit cluster density exhibited a significant increase at 14 dpi in the primary segment (G; mean ± SEM, ***P* < 0.01, Bonferroni *post-hoc* test) and remained unchanged distally (G').

In contrast to migration, overexpression of GFP-CB1$_{SH3-}$ enhanced dendritic arborization compared to GFP-control neurons. While the effect was not seen at 14 dpi – indicating that initial differentiation is not affected by eGFP-CB1$_{SH3-}$ – it was significant at 28 dpi and was most pronounced at 42 dpi (Mann-Withney U-test; Fig. 1C-C''). As seen in the graphs, the effects of eGFP-CB1$_{SH3-}$ were most prominent on distal dendrites (>100 μm from the soma), suggesting that the effect is on elongation of terminal branches. Accordingly, analysis of total dendritic length revealed a significant effect of time $F_{(2, 66)}$ = 52.70, P = 0.0001) and retrovirus construct $F_{(1, 66)}$ = 10.24, P = 0.021), as well as a significant interaction ($F_{(2, 66)}$ = 5.105, P = 0.087). Post-hoc analysis confirmed that CB1$_{SH3-}$ over-expression increased dendritic length by about 30% at 28 and 42 dpi (Fig. 1D).

As the main effect of CB overexpression in primary neuron cultures is to increase the size and density of gephyrin clusters, we were particularly interested to see if this effect can be reproduced *in vivo*. To distinguish between proximal and distal GABAergic synapses, the density of gephyrin clusters on the primary dendritic segment (up to the first bifurcation, typically located inside the GCL) and on secondary/tertiary segments located in the ML) was counted separately. Much to our surprise, we observed no effect of constitutive GFP-CB1$_{SH3-}$ overexpression on gephyrin clustering in proximal dendrites. A moderate increase was evident in distal dendrites at 28 dpi, confirmed by two-way ANOVA (time: $F_{(2, 140)}$ = 7.307, P = 0.001; retrovirus construct: $F_{(1, 140)}$ = 6.721, P = 0.011; Fig. 1E-E'). Therefore, constitutive availability of GFP-CB1$_{SH3-}$ does not appear to be sufficient for strongly increasing GABAergic synapses; alternatively, homeostatic mechanisms might be operating *in vivo*, preventing excessive synaptic inhibition to occur, which might interfere with normal cell development. To test this possibility, we also quantified the density of GABA$_A$R α2 and α1 subunit clusters, which represent the two main GABA$_A$Rs mediating synaptic inhibition in GCs.

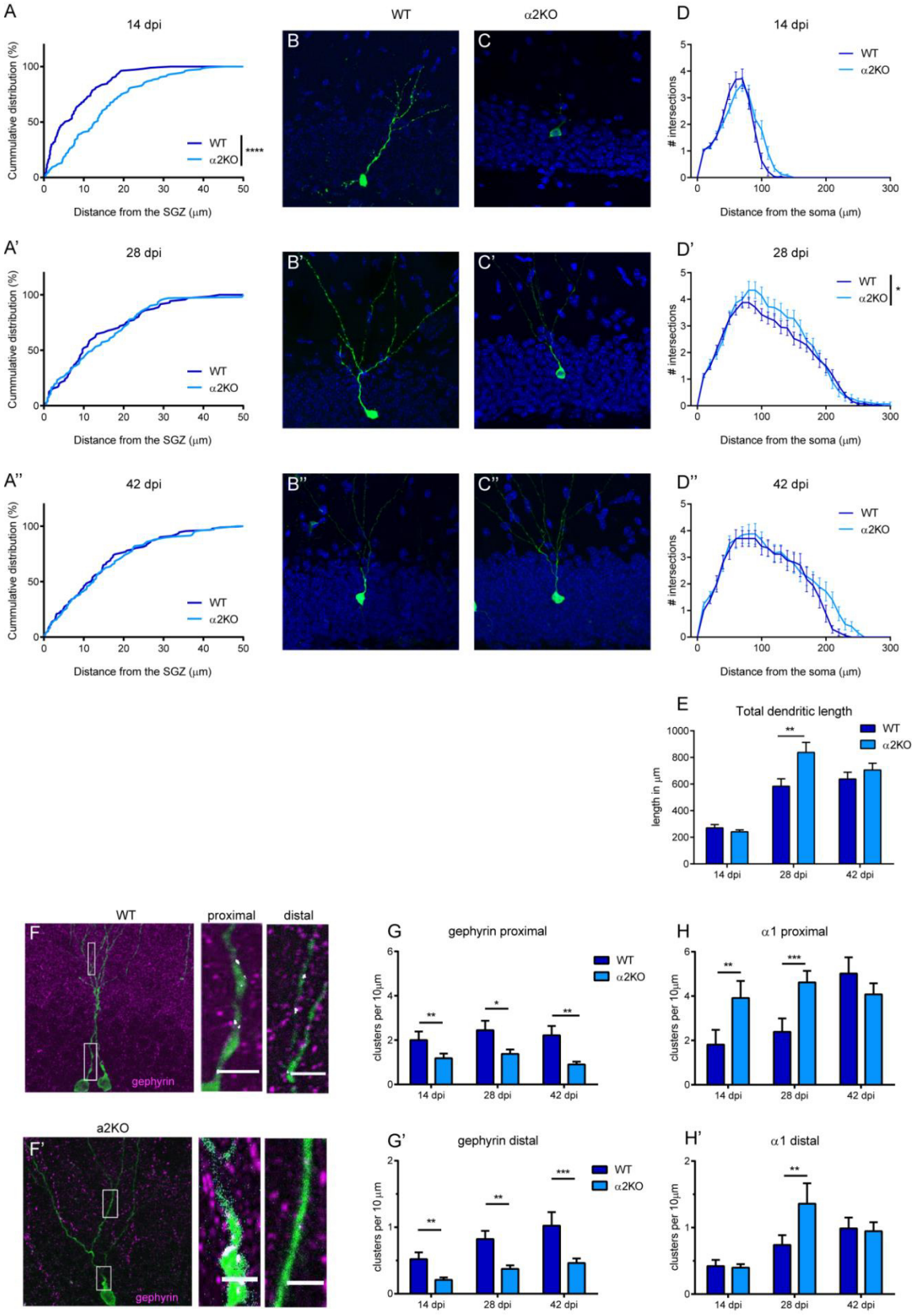
In line with the gephyrin results, this analysis revealed that GFP-CB1$_{SH3-}$ overexpression has no effect on the formation of α2 subunit clusters (Fig. 1F-F'), while transiently increasing the density of α1 subunit clusters at 14 dpi, selectively on proximal dendrites (two-way ANOVA, time: $F_{(2, 144)}$ = 17.25, P < 0.001; retrovirus construct: $F_{(1, 144)}$ = 7.464, P = 0.007; Fig. 1G-G'). The transient nature of this effect

again favors the existence of homeostatic mechanisms controlling GABAergic synapse formation despite overexpression of GFP-CB1$_{SH3-}$. In particular, it is conceivable that the elongation of dendrites leads to compensatory increase in α1-GABA$_A$R expression to balance neuronal excitability.

Taken together, the results reveal that constitutive overexpression in adult wild-type mice of a CB isoform having a short half-life causes only minor effects in adult-born GCs, mostly related to cytoskeletal function (motility, dendrite elongation). Therefore, we wondered whether CB overexpression might induce more changes in a disturbed context, and tested its effects in α2-KO mice, shown previously to exhibit major alterations in GC migration and differentiation (Duveau et al., 2011).

CB overexpression restores gephyrin clustering in Gabra2-null mice

Given that the α2 subunit has been shown to directly interact with CB isoform(s), we wanted to examine the functional relevance of this interaction in the context of adult neurogenesis. To this end, we first replicated our previous work in α2-KO mice and extended it to the analysis of α1 and α2 subunit clustering, as well as gephyrin clustering, in adult-born GCs of mutant mice, using a retrovirus encoding eGFP only. Measuring the migration distance of eGFP-transduced GCs from the SGZ, we observed that GCs in mutant mice penetrate deeper than wild-type control into the GCL at 14 dpi, but this difference subsided at later time-points (Kolmogorov-Smirnov test; Fig. 2A-A''). Furthermore, unlike our previous work, where we had observed significant pruning of dendrites between 28 and 42 dpi in α2-KO mice, we observed here by Sholl analysis only minor differences between genotypes, most pronounced at 28 dpi (Fig. 2D-D''), where GCs from mutant mice had slightly more complex dendritic trees, with increased total length (two-way ANOVA, retrovirus construct: $F_{(1,97)} = 4.729$, $P = 0.032$; Fig. 2E). In the present study, mice were housed in groups also after retrovirus injection, whereas in our previous work, they were single-caged, suggesting a possible role for stress-reducing measures on adult-born GC maturation to explain the differences between the two sets of results.



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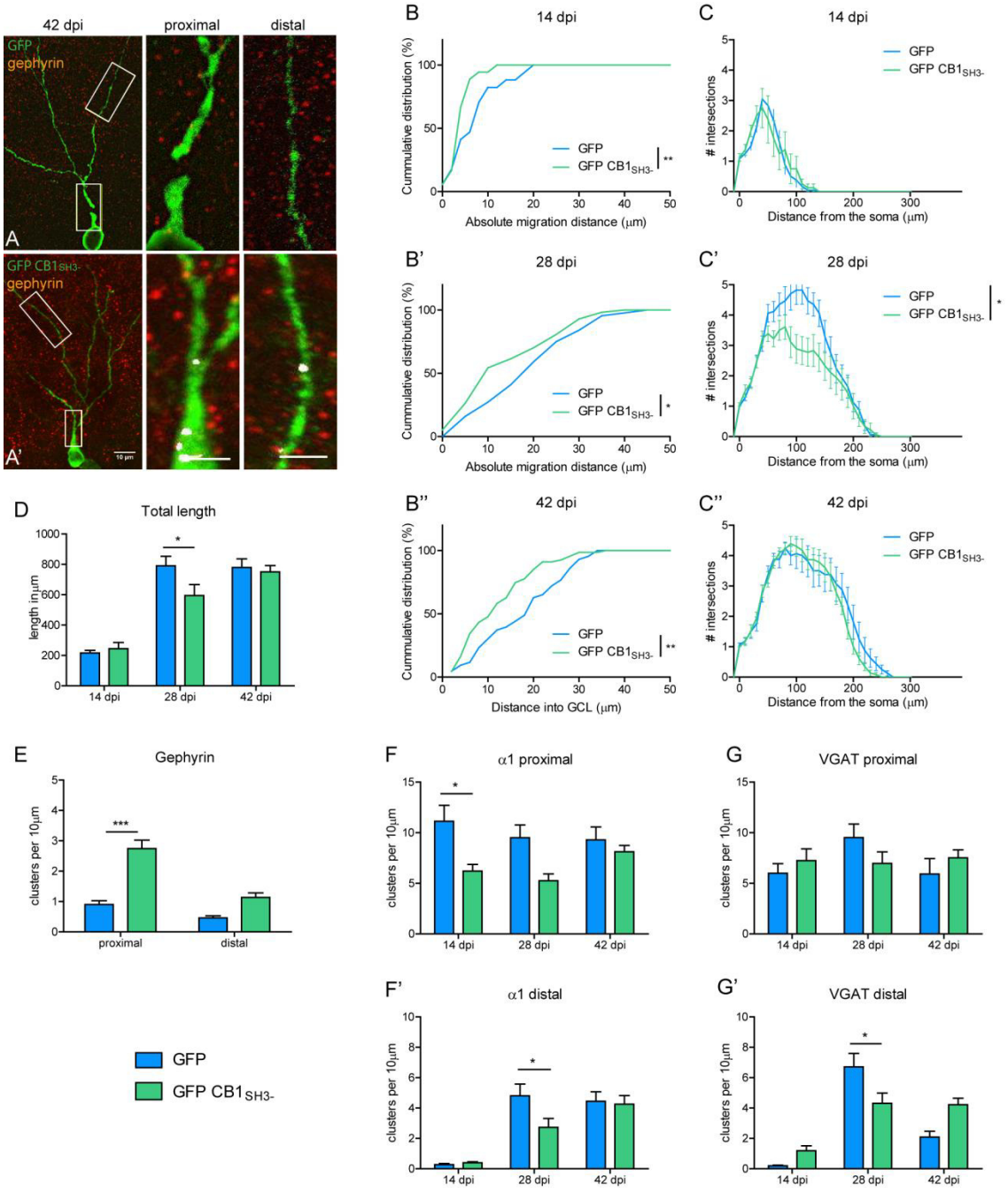
In CA1 pyramidal cells of adult α2-KO mice, there is a strong reduction of gephyrin postsynaptic clustering, underscoring the role of $GABA_AR$ for formation of the postsynaptic gephyrin scaffold (Panzanelli et al., 2011). Here, we extend this finding by showing by double immunofluorescence staining that it also applies to adult-born GCs, which, at each time-point examined, exhibited a significantly reduced density of gephyrin clusters on both proximal (two-way ANOVA, retrovirus construct: $F_{(1,203)}$ = 33.18, $P < 0.0001$) and distal dendritic segments (two-way ANOVA, retrovirus construct: $F_{(1,202)}$ = 40.01, $P < 0.05$; Fig. 2G-G'). The presence of residual gephyrin clusters points to other $GABA_AR$ subtypes in these cells; therefore, we quantified clusters formed by the α1 subunit and observed an almost 2-fold increase in immature cells. In proximal dendrites, the increase occurred at 14 and 28 dpi) and subsided thereafter (two-way ANOVA, time: $F_{(2, 151)}$ = 15.32, $P < 0.0001$; retrovirus construct: $F_{(1,151)}$ = 9.027, $P = 0.031$). Distally, only a significant time-dependent effect was seen ($F_{(2,153)}$ = 3.963, $P < 0.0001$; Fig. 2H-H'), with a significant difference occurring at 28 dpi. This increase likely represents a compensatory mechanism contributing to maintain fast synaptic inhibition in adult-born GCs, potentially explaining the moderate morphological phenotype observed in the Sholl analysis.

Figure 2. **Differences in adult-born GCs between wild type and α2-KO mice.** ***A.*** Cumulative distribution analysis of the migration distance covered by virally-transduced GCs at 14 (A), 28 (A') and 42 dpi (A''), showing a significant difference at 14 dpi (Kolmogorov–Smirnov test, ****$P <$ 0.0001). ***B, C.*** Representative images of GFP-positive GCs used for analysis of migration and dendritic arborizations at 14, 28 and 42 dpi, counterstained with DAPI (B-B": wild-type; C-C: α2-KO). ***D.*** Quantification of dendritic arborization by Sholl analysis did not show any significant difference in the area-under-the-curve between wild-type and α2-KO mice 14 and 42 dpi (D, D"), but a slight increase in α2-KO at 28 dpi (mean ± SEM *$P < 0.05$, Bonferroni *post-hoc* test). ***E.*** Analysis of the total dendritic length confirming the significant increase in mutant mice at 28 dpi (*$P < 0.05$, Bonferroni *post-hoc* test). ***F.*** Double immunofluorescence analysis of gephyrin clusters (magenta) on eGFP-positive dendrites in wild-type (F) and α2-KO mice (F'); the framed areas are enlarged on the right to display the localization of gephyrin (white) on eGFP-positive segments. Scale bar, 5 μm. ***G, G'.*** Quantification of gephyrin and α1 subunit cluster density on GFP-positive dendrites in wild-type and α2-KO mice at 14, 28 and 42 dpi. A significant reduction was observed in α2-KO mice at all time-points (mean ± SEM, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, Bonferroni *post-hoc* tests). ***H, H'.*** α1 subunit cluster density was increased at 14 and 28 dpi in the primary segment in α2-KO mice (H; mean ± SEM, **P<0.01; ***P<0.001; Bonferroni *post-hoc* tests), but not thereafter; in the distal dendrites (H'), this effect was seen only at 28 dpi (*$P < 0.05$).

Next, we examined whether overexpression of eGFP-CB1$_{SH3-}$ in adult-born GCs of α2-KO mice would differentially influence their maturation and synaptic integration compared to wild-type mice shown in Figure 1. We observed, unexpectedly, that unlike in wild-type mice, where eGFP-CB1$_{SH3-}$ overexpression increased GC migration, it caused a marked decrease in migration in α2-KO mice, in particular at 14 dpi when no GC migrated further than 10 μm into the GCL (Fig. 3B). Even at 28 and 42 dpi, when the maximal migration distance was the same for eGFP- and eGFP-CB1$_{SH3-}$-transduced cells, the deficit remained significant (Fig. 3B'-B''), as confirmed statistically (Kolmogorov-Smirnov). An opposite effect of eGFP-CB1$_{SH3-}$ overexpression between wild-type and α2-KO mice was seen also in relation to the regulation of dendritic growth. In the mutant, eGFP-CB1$_{SH3-}$ reduced dendritic complexity and length at 28 dpi (two-way ANOVA, time: $F_{(2, 113)} = 53.22$, $P = 0.0001$; retrovirus construct: $F_{(1,113)} = 4.598$, $P < 0.034$; Fig. 3C-C'', 3D), contrasting with the increase seen in wild-type mice. Taken together, these findings indicate that CB-mediated regulation of cell motility and dendrite branching depends on signaling mediated at GABAergic synapses, either by α2-GABA$_A$Rs or by signaling molecules interacting with the gephyrin scaffold.

Figure 3: Effects of CB overexpression in α2-KO mice. ***A,A'.*** Double immunofluorescence analysis of gephyrin clusters (red) on dendrites expressing eGFP-only (A) or eGFP CB1$_{SH3-}$ (A'); the framed areas are enlarged on the right to display the localization of the clusters (white) on eGFP-positive segments. Scale bar, 5 μm. ***B.*** Cumulative distribution analysis of the migration distance covered by virally-transduced GCs at 14 (B), 28 (B') and 42 dpi (B'') in α2-KO mice. A significant reduction was evident in GCs overexpressing eGFP-CB1$_{SH3-}$ compared to eGFP control at all time-points investigated (Kolmogorov–Smirnov test, *$P < 0.05$, **$P < 0.01$). ***C.*** Quantification of dendritic arborization of eGFP and eGFP-CB1$_{SH3-}$ GCs by Sholl analysis at 14 (C), 28 (C') and 42 (C'') dpi in α2-KO mice. eGFP-CB1$_{SH3-}$ overexpression caused a significant decrease in the AUC at 28 dpi (Mann-Whitney *t*-test, *$P < 0.05$). ***D.*** Analysis of the total dendritic length revealed a significant increase in GCs expressing eGFP-CB1$_{SH3-}$ at 28 dpi only (*$P < 0.05$, Bonferroni *post-hoc* test). ***E.*** Quantification of gephyrin cluster density at 42 dpi revealed a strong increase in the proximal segment of GCs expressing eGFP-CB1$_{SH3-}$ (mean ± SEM, ****$P < 0.0001$, Bonferroni *post-hoc* test); in distal dendrites the effect was variable and did not reach statistical significance. ***F, G.*** Quantification of the density of α1 subunit clusters and vGAT+- terminals on dendrites of transduced GCs at 14, 28 and 42 dpi. The density of α1 subunit clusters was reduced in proximally at 14 dpi by eGFP-CB1$_{SH3-}$ overexpression (F; mean ± SEM, ***$P < 0.001$, Bonferroni *post-hoc* test) and distally at 28 dpi (F'; mean ± SEM, *$P < 0.05$, Bonferroni *post-hoc* test). The density of vGAT+- terminals remained unchanged proximally (G), whereas distally, it paralleled the decrease in α1 subunit clusters at 28 dpi (G'; mean ± SEM,*$P < 0.05$, Bonferroni *post-hoc* test).



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Therefore, we tested whether eGFP-CB1$_{SH3-}$ overexpression in GCs of α2-KO mice affected gephyrin and α1-GABA$_A$R clustering. Quantification of gephyrin cluster density at 42 dpi revealed a pronounced increase compared to control cells expressing eGFP-only (two-way ANOVA, $F_{(1, 117)} = 16.62$, $P < 0.0001$; Fig. 3E); when compared to wild-type cells (Figure 2), gephyrin clustering was restored to normal levels. Interestingly, the density of α1 subunit clusters was decreased by eGFP-CB1$_{SH3-}$ at 14-28 dpi (two-way ANOVA, proximal, retrovirus construct: $F_{(1,88)} = 9.493$, $P = 0.003$; distal, retrovirus construct, $F_{(1,92)} = 4.334$, $P = 0.04$; Fig. 3F-F'), reversing the putative compensatory increase seen in α2-KO mice. Finally, eGFP-CB1$_{SH3-}$ overexpression had no consistent effects on the number of vGAT-positive terminals apposed to proximal or distal dendritic segments of adult-born GCs, although a significant decrease was observed in distal dendrites at 28 dpi (two-way ANOVA, retrovirus construct: $F_{(1, 84)} = 5.114$, $P = 0.02$; Fig. 3G-G').

The results so far indicate that eGFP-CB1$_{SH3-}$ causes mixed effects on maturation and synaptic integration of adult born GCs, independence of the integrity of GABAergic synapses and possibly reflecting the multiple functions of CB for the regulation of gephyrin, as well as Cdc42 and TC-10 activity. Therefore, to better understand the mechanisms underlying the mixed effects observed so far, we tested the consequences of chronic down-regulation of all CB isoforms, selectively in adult-born GCs of wild-type mice, using retrovirus-mediated transduction of eGFP together with CB shRNA.

CB down-regulation favors dendrite growth without affecting GABAergic synapse formation

To test the effectiveness of the shRNA to down-regulate CB, we transfected HEK-293T cells with rat V5-CB1$_{SH3+}$ or V5-CB1$_{SH3-}$ together with four different shRNA constructs and a scrambled RNA sequence. The shRNAs were directed against (1) the SH3 domain of CB, (2) a coding sequence conserved in all CB isoforms across species, and (3, 4) two coding sequences selective for mouse CB. Three days after transfection, Western Blot analysis of cell extracts showed a significant, specific reduction of CB with the shRNAs Nr. 1 and 2 (CB-specific SH3 domain and panCB); the shRNAs Nr. 3 and 4 (directed against mouse CB) were only moderately effective (Fig. 4A). To further validate the panCB-shRNA, we transfected cultured rat

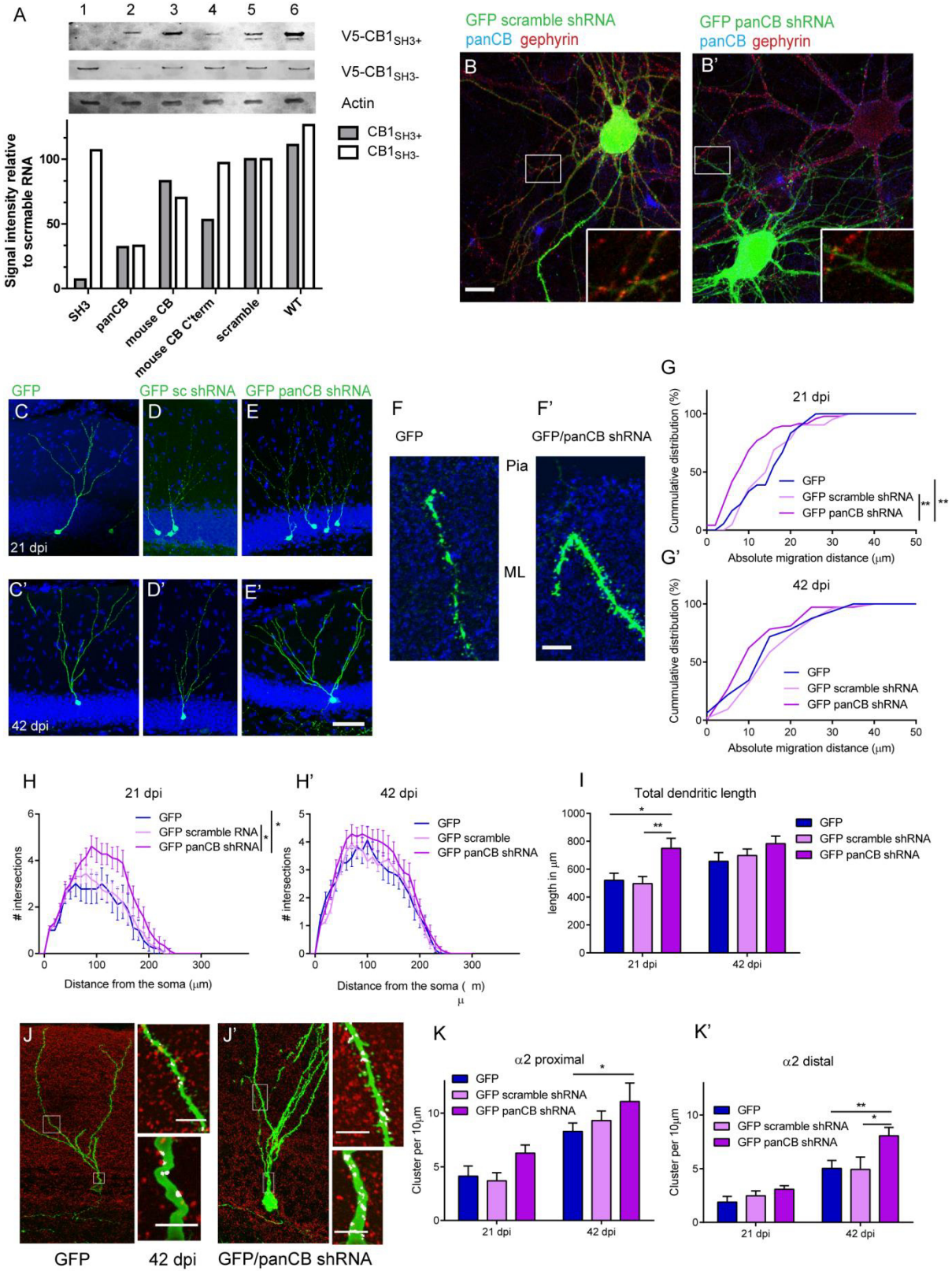
hippocampal neurons at 12 days *in vitro* (DIV) with eGFP together with panCB-shRNA or scrambled-RNA and stained the cultures 7 days after transfection for endogenous CB and gephyrin. Despite of the strong GFP expression, the neurons looked healthy and a selective loss of CB and gephyrin clusters could be seen in cells transfected with eGFP and panCB shRNA. Expression of the scrambled sequence had no effect compared to untransfected neurons in the same coverslip (Fig. 4B-B'). Thus, the panCB shRNA effectively reduced CB expression without inducing signs of toxicity in this assay.

Adult wild-type mice were injected with retroviruses encoding eGFP, eGFP and scramble-RNA or eGFP and panCB-shRNA and analyzed at 21 and 42 dpi. At first glance, especially at 42 dpi, major effects of the panCB-shRNA were evident, as the cells had longer and more complex dendrites (Fig. 4C-E). We even observed dendrites that reverted their radial course when reaching the outer border of the ML, in both the upper and lower blades of the DG (Fig. 4F-F'). Measuring the migration distance from the subgranular zone, revealed unexpectedly that silencing all CB isoforms with the panCB-shRNA construct had the same effect as over-expressing eGFP-CB1$_{SH3-}$, namely to reduce the migration of GCs during early phases of maturation (one-way ANOVA, $F_{(2,105)} = 7.409$, $P < 0.001$; Fig. 4G-G'). Thus, this result suggests that the effects of CB on cell motility are mediated indirectly. The marked enhancement of dendritic branching and growth, observed visually, was confirmed by Sholl analysis, which revealed almost a 40% increase in total dendrite length at 21 dpi compared to both control cell populations (two-way ANOVA, time: $F_{(1, 104)} = 7.088$, $P = 0.09$; retrovirus construct: $F_{(2,104)} = 6.422$, $P = 0.023$). At 42 dpi, the effect on branching had subsided and total dendritic length was similar to both controls (Fig. 4H-H',4I).

Finally, we tested whether CB down-regulation affected the density of α2 subunit clusters on the proximal and distal dendrites. A significant increase in α2 subunit clusters occurred at 42 dpi in both proximal and distal dendrite segments (two-way ANOVA, proximal, retrovirus construct: $F_{(2,58)} = 3.279$, $P = 0.045$; distal, retrovirus construct: $F_{(2,58)} = 5.94$, $P = 0.0045$; Fig. 4K-K'), standing in striking contrast with the loss of α2 subunit clusters reported in CB-KO mice (Papadopoulos et al., 2007).

Therefore, down-regulation of CB in adult-born neurons maturing in a normal environment has different effects than its targeted abrogation in CB-KO mice.

Figure 4. CB down-regulation favors dendrite growth without affecting GABAergic synapse formation*. A.* Quantitative Western blot analysis of CB levels in HEK-293T cells co-transfected with rat V5-$CB1_{SH3+}$ or V5-$CB1_{SH3-}$ and a plasmid encoding eGFP and shRNAs directed against (1) the SH3 domain of CB, (2) a coding sequence of CB conserved in all CB isoforms across species, (3, 4) two coding sequences selective for mouse CB; as control, a scrambled shRNA sequence (5) or only V5-CB1 isoforms were expressed. The relative protein amount was determined from the intensity of the protein bands, using actin as a standard, and normalized to the values of the scramble shRNA. The shRNA against CB SH3 domain caused a specific reduction of $CB1_{SH3+}$ and panCB shRNA equally affected both CB1 isoforms; the shRNA directed against mouse CB were ineffective, and scrambled shRNA expression was similar to control, indicating no toxic effects of the shRNA constructs. ***B.*** Images of neurons co-transfected with eGFP and panCB-shRNA (B) or scrambled-RNA (B') and stained for endogenous CB (blue) and gephyrin (red), demonstrating the reduction in CB staining as well as gephyrin clustering induced by the panCB-shRNA. ***C-E.*** Representative images of GCs transfected with eGFP only (C,C'), eGFP and scrambled shRNA (D,D'), or eGFP and panCB shRNA (E,E') at 21 and 42 dpi (scale bar, 25 μm). **F, F'.** Illustrations of dendrites reaching the border of the ML; pan-CB silencing caused further growth in the reverse direction (scale bar, 5 μm). ***G, G'.*** Cumulative distribution analysis of the migration distance covered by eGFP+ cells at (G) 21 and (G') 42 dpi. Relative to both controls, a significant reduction was evident at 21 dpi in panCB shRNA expressing GCs (**P*<0.01; ***P* < 0.001, Kolmogorov-Smirnov test). ***H, H'.*** Quantification of dendritic arborization by Sholl analysis at 21 and 42 dpi, showing the significant increase in the area-under-the-curve caused by CB silencing (**P* < 0.05, Bonferroni *post-hoc* test). ***I.*** Analysis of the total dendritic length confirmed the increase in GCs expressing panCB shRNA at 21 dpi only (**P*<0.05; ***P* < 0.01, Bonferroni *post-hoc* test). ***J, K.*** Images depicting the distribution of α2 subunit clusters (red) on eGFP-positive dendrites of control (J) and eGFP/panCB shRNA-expressing GCs (J'). Boxed areas are enlarged on the right to illustrate α2 subunit clusters (white) localized on the GFP-positive dendrite (scale bar, 5 μm). ***K.*** Quantification of α2 subunit clusters co-localized with GFP/panCB shRNA revealed an increase in the proximal (mean ± SEM, **P* < 0.05, Bonferroni *post-hoc* test) and distal segments (***P* < 0.01) at 42 dpi compared to control groups.



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CB down-regulation alters spine formation and maturation

Spine formation in adult-born GCs occurs mainly during the third and fourth weeks post-final division, providing postsynaptic sites for incoming glutamatergic synapses. It has been reported that over-expression of a dominant negative Cdc42 construct leads to an increase in immature spines around this age (Vadodaria et al., 2013). Therefore, we examined spine formation and morphology in high-magnification images of eGFP immunofluorescence in control GCs and upon transduction with panCBshRNA (Fig. 5). Spine quantification in GCs expressing GFP/panCB shRNA revealed no difference in the overall spine density compared to control at 21 dpi (Fig. 5E). Classification of spines according to their morphology (thin, stubby, and mushroom) likewise revealed no difference among the three groups at this stage (Fig. 5A-B). In contrast, eGFP/panCB-shRNA expression led to a marked increase selectively in immature spines at 42 dpi (two-way ANOVA, retrovirus construct: $F_{(1,.47)} = 4.319$, $P = 0.043$) (Fig. 5F), which in total represented close to 50% of total spines (Fig. 5C- D).

As this result mimics the effects of silencing Cdc42 with a dominant-negative construct, it raises the question how Cdc42 activation in spines is affected in the absence of CB.

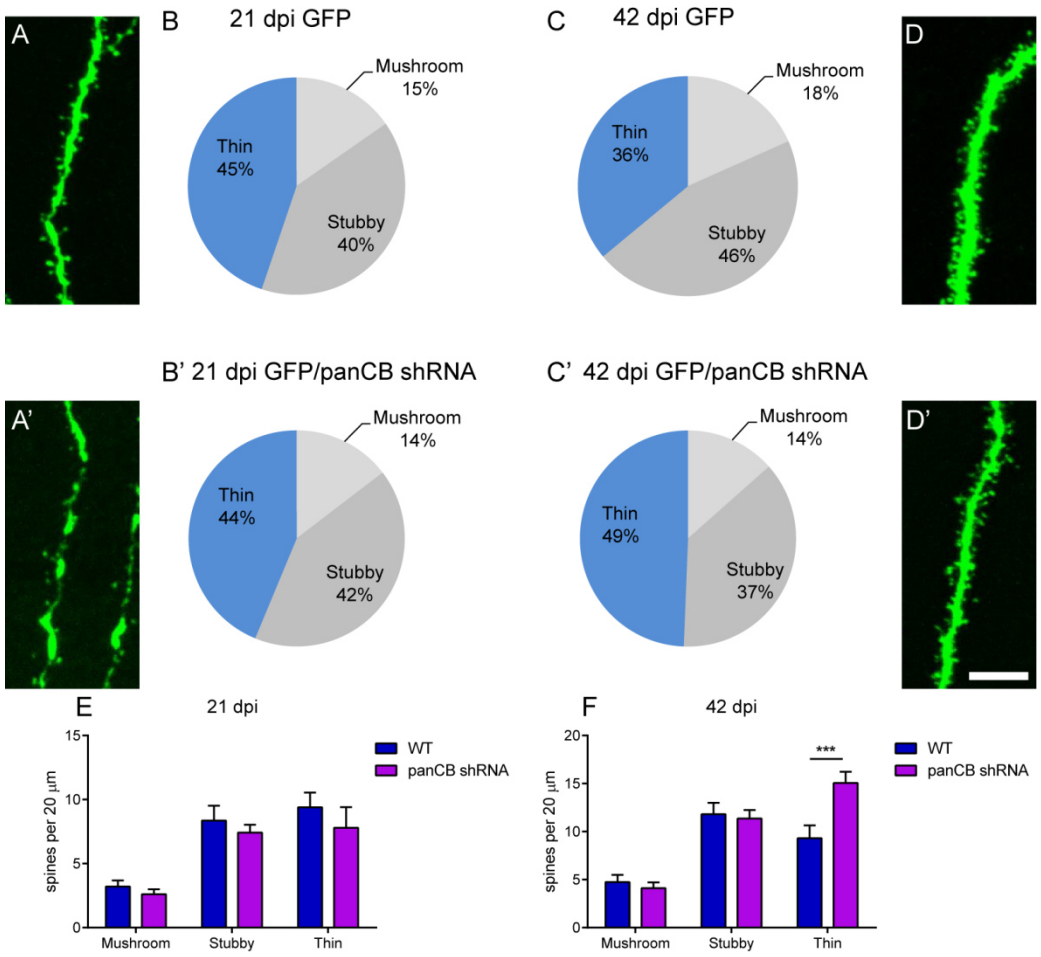

Figure 5. **CB down-regulation alters spine formation and maturation.** ***A, D.*** Representative images of dendrite segments from eGFP- (A, D) and eGFP/panCB shRNA- (A', D') expressing GCs at 21 dpi and 42 dpi (scale bars, 5 µm). ***B C.*** Relative abundance of spines according to their morphology (mushroom, stubby, or thin spines). CB-silencing had no effect on proportion of thin spines at 21 dpi, but it caused a strong increase at 42 dpi, without affecting the distribution of stubby and mushroom spines. ***E F.*** Quantification of spine density, separately for mushroom, stubby and thin spines at 21 and 42 dpi. CB-silencing caused a significant increase in thin spines density at 42 dpi (mean ± SEM, ***$P < 0.001$, Bonferroni *post-hoc* test), explaining their higher relative abundance.

DISCUSSION

The results of this study demonstrate that overexpression and suppression of CB in adult-born dentate gyrus GCs markedly affects their migration from the SGZ and increases their dendritic differentiation, while having only moderate effects on gephyrin and $GABA_AR$ $\alpha1$ or $\alpha2$ subunit postsynaptic clustering. Strikingly, in wild-type mice, $CB1_{SH3-}$ overexpression and shRNA-mediated down-regulation of all CB isoforms had similar promoting effects on dendrite arborization, suggesting that both conditions can activate signaling factors contributing to dendritic growth, such as the small Rho-GTPase Cdc42 (or possibly TC10). This activation is spectacularly demonstrated by the failure of dendrites to stop growing when they reach the outer surface of the molecular layer (Fig. 4F-F'). This hypothesis implies, however, that CB down-regulation leads to a long-lasting activation of Rho-GTPases.

A second unexpected result is the increase in density of $\alpha2$ subunit clusters, notably in distal dendrites, upon down-regulation of CB isoforms, which starkly contrasts with the region-specific loss reported in adult CB-KO mice (Papadopoulos et al., 2007). A possible reason for this discrepancy is the incomplete silencing of CB achieved by the shRNA construct, as shown upon transient transfection is HEK-293 cells (Fig. 4A). Alternatively, as Cdc42 has been shown to rescue gephyrin clustering when CB is functionally inactivated (Tyagarajan et al., 2011a), it is conceivable that CB silencing strongly activates Cdc42 and thereby prevents the loss of GABAergic postsynaptic markers seen upon constitutive deletion.

Finally, the results unravel a role of CB in controlling dendritic spine maturation in differentiated GCs. The increased proportion of immature spines on GC dendrites at 42 dpi is once again in line with a constitutive activation of Cdc42 upon CB down-regulation. Therefore, this effect might reflect the existence of a mechanism through which CB, via the control of Cdc42 activity, contributes to inhibitory/excitatory balance in mature GCs by setting a break on spine plasticity and turnover.

Taken together, the results unravel novel functions of CB for the regulation of neuronal maturation and disclose that CB-mediated activation of Cdc42 is likely subjected to a complex regulation that can be disrupted by either overexpressing or silencing CB in neurons.

Overexpression of CB in wild-type mice affects GC migration and dendritic arborization

First evidence *in vivo* of a role for $GABA_AR$-mediated transmission in regulating migration of adult-born dentate gyrus granule cells came from the analysis of α4-KO mice, in which a pronounced deficit in migration was observed, as the majority of cells remained within or close to (<5 μm) the SGZ (Duveau et al., 2011). Here, we show that CB-dependent signaling likewise affects the positioning of new granule cells. Overexpression of $CB1_{SH3-}$ caused a ~10 μm leftward shift of the cumulative probability distribution curve (Fig. 1B-B"), indicating that these GCs migrate a shorter distance into the GCL during their maturation. The effect was even more prominent in α2-KO mice, where migration of adult-born GCs can exceed that seen in wild-type mice, indicating that the migration deficit is not solely due to defective GABAergic transmission. However, GC migration was also affected in wild-type mice upon shRNA-mediated CB down-regulation, suggesting defective activation of signaling molecules mediating actin remodeling for cell motility. Therefore, CB levels need to be properly balanced, along with signals mediated via α4- and α2-$GABA_ARs$, to ensure proper adult-born GC final positioning within the GCL. Of note, the differential positioning of transduced GCs cannot be explained by differences in neural precursor cell proliferation or neurogenesis, as these parameters are not affected by the retrovirus injections or the *Gabra2* inactivation (Duveau et al., 2011).

Similar to migration, we observed that both $CB1_{SH3-}$ overexpression and pan-CB down-regulation increased dendrite growth and branching, albeit with different time-courses. Increasing $CB1_{SH3-}$ levels gradually leads to increased dendritic growth, in line with a possible stimulation of down-stream signaling via Cdc42. Silencing CB enhances dendritic growth at early time-points (21 dpi) and the effect subsides later on. This result is in line with a study demonstrating that inhibiting Cdc42 by overexpression of a dominant-negative mutant reduces dendrite growth in immature granule cells, but has no effect in mature cells (in which dendrite growth is regulated by Rac1) (Vadodaria et al., 2013). One parsimonious explanation for the activation of Cdc42 upon CB silencing might be that CB is a poor activator of Cdc42, and might even sequester it and prevent its activation by more effective GEFs. According to this line of thinking, the interaction between CB, gephyrin, and Cdc42,

which can form ternary complexes (Tyagarajan et al., 2011a), might represent a regulatory mechanism allowing Cdc42 activation only at the vicinity of GABAergic synapses. Perturbing such a complex by either CB over-expression or silencing might result in uncontrolled Cdc42 activation.

CB-dependent and independent regulation of gephyrin and $GABA_AR$ clustering

In cultured hippocampal neurons, we have observed that overexpression of $CB1_{SH3-}$ between 8 and 15 DIV causes a significant increase in postsynaptic gephyrin cluster density (De Groot et al., in preparation); the effect is more pronounced with CB isoforms that have a longer half-life, indicating that CB might stabilize gephyrin and favor its postsynaptic clustering, in line with previous studies (Chiou et al., 2011; Tyagarajan et al., 2011a; Körber et al., 2012). This effect is not readily seen here *in vivo* in adult-born GCs, suggesting that, in the normal physiological context, the effects of CB on gephyrin might be buffered by homeostatic mechanisms preventing excess formation of GABAergic synapses. Another possible rate-limiting factor might be the availability of presynaptic terminals to form supernumerary synapses. However, because $CB1_{SH3-}$ caused a pronounced increase in dendritic growth, evidenced both in the complexity of the dendrite arbor and its total length, the absolute number of gephyrin clusters (representing presumptive postsynaptic sites) is increased in these cells. Likewise, silencing CB expression had little effect on $\alpha2$ subunit clustering, except at 42 dpi, where a significant increase was observed in distal dendrites.

Furthermore, the quantification of postsynaptic $GABA_AR$ clusters revealed in control mice an initial predominance of the $\alpha2$ subunit, both in proximal and distal dendrite segments, with a delayed increase of the $\alpha1$ subunit. It is not known whether these subunits are present in distinct receptors, or perhaps even in distinct synapses. However, this temporal pattern reproduces the general trend of delayed of $\alpha1$-$GABA_AR$ expression occurring during ontogeny, suggesting that these receptors, which are characterized by fast decay kinetics (Brussaard et al., 1997), are a marker of mature neurons. The density of α subunit clusters was higher than gephyrin clusters. This difference might be explained by the presence of extra-synaptic $GABA_AR$ clusters or by technical limitations in detecting gephyrin immunoreactivity, possibly related to gephyrin phosphorylation, as reported (Kuhse et al., 2012).

Our results provide strong evidence that CB acts downstream, rather than upstream of $GABA_A$Rs to regulate postsynaptic gephyrin clustering, as suggested from *in vitro* studies (Poulopoulos et al., 2009; Saiepour et al., 2010). Thus, CB overexpression in GCs from α2-KO mice rescued the deficient gephyrin clustering in proximal as well as distal dendrites, but also prevented the compensatory upregulation of α1 subunit clusters. These results, which extend previous *in vitro* observations in neuron cultures from α2-KO and γ2-KO mice (de Groot et al., in preparation; and unpublished), show that CB's ability to increase gephyrin clustering does not require interaction with postsynaptic $GABA_A$Rs; further, they imply that the number of GABAergic synapses formed by adult-born GCs is tightly regulated independently of the levels of CB expression.

CB regulates spine formation in mature neurons

In adult-born GCs, dendritic spines start to form during the third week post-final mitotic division, marking the initiation of glutamatergic synapse formation. Spines are highly dynamic protrusions, which change shape as they mature and the excitatory synapses strengthen. Inhibiting Cdc42 activity *in vivo* by infecting newborn neurons with a dominant negative isoform of Cdc42 had no effect on spine formation or density, but affected their maturation (Vadodaria et al., 2013). Here, we show that CB down-regulation increases the formation of immature spines selectively in differentiated GCs. As the density of mushroom and study spines remained unchanged compared to control, we interpret this finding as an effect of Cdc42 activation. This observation may also explain the increase of α2 subunit clusters at 42 dpi in the distal dendrites, which often were localized in immature spines (Fig. 4J'). It is possible, of course, that the majority of such spines are only transient, their formation reflecting increased spine plasticity and turnover. Therefore, by means of locally-controlled activation of Cdc42, CB might represent an important regulator of the inhibitory/excitatory balance by inhibiting the formation of spines (and consequently glutamatergic afferents).

CB negatively regulates Cdc42

Current models of CB function in GABAergic synapses emphasize its interactions with gephyrin and its required membrane targeting via the PH domain to regulate gephyrin clustering (Reddy-Alla et al., 2010; Papadopoulos and Soykan, 2011).

Activation of Cdc42 is postulated to modify the actin cytoskeleton, possibly contributing the formation, stabilization, or plasticity of GABAergic synapses, but its precise role is unknown. Accordingly, the SH3 domain is viewed as a key regulator of CB function, enabling or disabling both membrane anchoring and enzymatic activity upon conformation changes (Poulopoulos et al., 2009; Mayer et al., 2013). Our results here show that overexpression of $CB1_{SH3-}$ phenocopies major effects of CB down-regulation, suggesting at first glance that $CB1_{SH3-}$ might act as a dominant-negative CB isoform. However, this possibility is unlikely, since $CB1_{SH3-}$ rescues gephyrin clustering in adult-born GCs of α2-KO mice and promotes dendritic growth in wild-type mice. Rather, we postulate that overexpression of $CB1_{SH3-}$ and panCB down-regulation both lead to increased Cdc42 activation by acting in the opposite manner on a common pathway. The constitutive Cdc42 activation apparently occurring in GCs transfected with the panCB shRNA construct strongly suggests that CB binding to Cdc42 might sequester it away from GEFs that have a stronger enzymatic activity. It is even conceivable that CB binding to gephyrin might be required for it to be able to activate Cdc42. While this possibility needs to be tested experimentally, it would explain why the three proteins form a complex and how CB ensures Cdc42 activity at GABAergic synapses (where it regulates the shape and size of gephyrin clusters (Tyagarajan et al., 2011a)). Moreover, this mechanism would explain why increased $CB1_{SH3-}$ expression might enhance Cdc42 activity, assuming that the excess CB protein interacts with gephyrin and becomes thereby enzymatically active. An alternative model posits that a conformational change in CB, which increases its binding affinity to PI3P in the plasma membrane, converts it from a close/inactive form into an open/active form, and that proteins, such as neuroligin2, which bind the SH3 domain of CB, favor its open conformation (Soykan et al., 2014). In the case of $CB1_{SH3-}$, it is conceivable that gephyrin binding might have a similar effect. According to this view, CB would be a key regulator of Cdc42 in neurons, remaining inactive until anchored to PI3P and bound to gephyrin. The short half-life of $CB1_{SH3-}$ might thus be viewed as a mechanism allowing dynamic control of Cdc42 activity as well, either by "freeing" binding sites on gephyrin for other CB isoforms or by allowing a rapid increase in CB when Cdc42 needs to be activated at GABAergic synapses.

Along the same line, CB binding to gephyrin might be crucial to regulate the formation of novel gephyrin clusters. Indeed, it has been suggested that phosphorylation of gephyrin S270 residue, which is a negative regulator of gephyrin cluster formation, by GSK3β and/or cdk-5 requires CB binding (Kuhse et al., 2012).

In conclusion, this study adds to the evidence that the molecular mechanisms contributing to the regulation of gephyrin postsynaptic clustering and, thereby, GABAergic function, also have profound influences on migration, morphological differentiation, and synaptic integration of adult-born neurons in the CNS.

ACKNOWLEDGMENTS

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IV. GENERAL DISCUSSION

CB is a major regulator of gephyrin postsynaptic clustering, and thereby, GABAergic synaptic function, as shown either by gene targeting studies or by neuronal over-expression *in vitro*. The dominating view, at the start of my PhD, was that the SH3 domain of CB controls its gephyrin clustering activity, and thereby the formation of GABAergic synapses. Although the existence of multiple splice variants of CB, differing only in their C'-terminal domain, was well established, the significance of this diversity had not been investigated. Likewise, although CB is a GEF selectively activating Cdc42, the relevance of this function of CB remained obscure. Therefore, the main aims of this thesis were to analyze the role of CB1 and CB2 isoforms for postsynaptic gephyrin clustering *in vitro* and to investigate the relevance of CB over-expression or down-regulation in the context of neuronal maturation and synaptogenesis *in vivo*, using adult neurogenesis as a model system and focusing on developmental steps known to be regulated by GABAergic transmission. Here, I will first summarize the main results of my two studies before discussing separately their implications in broader context.

The generation of CB-KO mice revealed that CB is essential for gephyrin and $GABA_AR$ clustering at GABAergic synapses, albeit in a cell- and region-specific manner (Papadopoulos et al., 2008). In the *in vitro* study, we could demonstrate in cultured neurons from γ2-KO mice that CB is able to rescue gephyrin clustering in the absence of postsynaptic $GABA_AR$, underscoring the importance of CB for postsynaptic gephyrin stabilization. Further, this study revealed that while CB isoforms have redundant functions, they undergo differential regulation by post-transcriptional and translational mechanisms in order to be to adapted for the needs of different GABAergic synapses and contribute to their structural and functional plasticity. In particular, we could show that chronic neuronal stimulation with KCl increases CB protein levels, while affecting splice site selection for CB isoforms, causing rapid changes in mRNA abundance of specific splice variants. Prolonged neuronal activity led to a reduction of CB1 and CB3 mRNA, whereas CB2 mRNA levels remained constant. Since overall mRNA transcript levels were unchanged, the

regulation occurred at the post-transcriptional level. In addition, we could inhibit this effect by blocking Ca^{2+}-calmodulin kinase signaling, suggesting that changes in CB splicing occur to maintain excitatory-inhibitory balance, possibly by favoring the rapid formation of additional GABAergic synapses.

Further evidence that CB1 might be specifically involved in transient synapse regulation comes from the observation that it has a much shorter half-life than CB2, which is determined post-translationally by ubiquitin-mediated degradation, acting on identified lysine residues in the C'-terminus. The importance of CB half-life was shown by the increase of gephyrin clusters in cultured primary neurons, when transfected with CB with mutated lysine residues compared to the native CB isoform.

Finally, using single RNA *in situ* hybridization with probes for panCB and CB1, we observed that CB mRNAs are transported in dendrites, where they likely are subject to local translation in the vicinity of GABAergic synapses.

Based on these results, we postulated in the *in vitro* study a model whereby CB1 would selectively contribute to formation and plasticity of transient GABAergic synapses, established in response to elevated neuronal activity, whereas CB2 might be associated with stable GABAergic synapses, ensuring long-term maintenance of post-synaptic gephyrin and $GABA_AR$ clusters

The *in vivo* study revealed a novel unexpected role of CB. The overexpression or silencing of CB showed an effect on cell motility, cytoskeletal organization, dendritic length and spine maturation. We demonstrated that the increase in dendritic length and the maturation of spines was not due to a direct activation of Cdc42 (or TC10) by CB, but rather a negative regulation, whereby CB might be sequestering Cdc42 away from other RhoGEFs. This hypothesis was underlined by the inability of dendrites in WT mice with silenced CB expression to stop growing, when they reached to the outer border of the ML. In addition, the increase in immature spines in adult-born GCs with silenced CB expression suggests a role for CB in regulating glutamatergic synaptogenesis through the control of Cdc42 activity, contributing to the E/I balance.

Since GABA was shown to be involved in migration and maturation of neurons in the developing brain, our results provide a mechanism how GABA exerts these effects through signaling molecules associated with GABAergic synapses. Since overexpression of $CB1_{SH3-}$ caused the same effects as the down-regulation of CB on dendritic length, the regulation of CB through the interaction with the PSD of GABAergic synapse is essential. Furthermore CB overexpression did not induce an increase in gephyrin clusters in adult-born cells as it does in cultured neurons, indicating that CB is not a limiting factor for synapse formation in an intact network. However in α2-KO mice, CB overexpression rescued disrupted gephyrin clustering and normalized the density of $GABA_AR$ α1 subunit post-synaptic clusters. These results demonstrate that CB can substitute for postsynaptic $GABA_AR$ for ensuring gephyrin clustering. Since overexpression and silencing of CB affect the positioning of adult born GCs in the GCL and their dendritic development in similar ways, a tight regulation of CB is needed for proper Cdc42 activation, most probably through the binding to gephyrin at GABAergic synapses. This conclusion is in line with recent work showing that the conformation of CB is regulated by multiple mechanisms involving several major domains of the protein that interact with molecules of the GABAergic PSD, as well as membrane phosphoinositides (Soykan et al., 2014).

Taken together, our studies demonstrate that CB is a key regulator of GABAergic synaptic plasticity, based on the differential use of its splice variants under the control of activity-dependent mechanisms. Further, CB has strong structural effects on neuronal development by means of gephyrin-dependent regulation of Cdc42 activity. The latter finding provides a mechanism explaining how GABAergic transmission, and in particular the formation of GABAergic synapses, is a key regulator of neuronal migration, dendritic maturation, spine formation, and synaptic integration.

The importance of CB isoforms for GABAergic synaptic plasticity

Two forms of GABAergic synaptic plasticity have been reported in the literature. First, following long-term sensory deprivation, for example, increased GABAergic synapse turnover has been reported in primary sensory cortical areas (Levelt and Hübener, 2012; Chen and Nedivi, 2013), which occurs to compensate for corresponding changes in glutamatergic synaptic connections. Similar structural

reorganization might also occur following learning to stabilize newly formed circuits, possibly underlying procedural memory. Remarkably, changes in GABAergic connectivity also occur, at least *in vitro*, upon inhibition of signaling cascades by lithium chloride (Tyagarajan et al., 2011b), or following increased metabolism and oxidative stress (Accardi et al., 2014). Second, rapid adaptations in synaptic strength, potentiation or depression, occur following Hebbian paradigms of plasticity, such as, for example, rebound potentiation of GABAergic currents in Purkinje cells following coincident depolarization and stimulation of parallel fiber input (Kano, 1994). These forms of homeostatic GABAergic plasticity involve changes in the function and number of postsynaptic $GABA_AR$, mostly through recycling of the receptor, lateral diffusion, or phosphorylation. It is tempting to speculate that both forms of plasticity require adaptations of the gephyrin scaffold and are made possible by the integration of the effector pathways in the PSD (Petrini and Barberis, 2014). Importantly, it has been shown that activity-dependent increase in amplitude of synaptic currents is directly correlated with the number of $GABA_AR$ present in the synapse and the size of the PSD (Nusser et al., 1998). Since CB influences the number and size of gephyrin clusters, it is directly linked to GABAergic plasticity. The results of the *in vitro* study showed that CB1 is a short-lived isoform and is regulated by neuronal activity, whereas CB2 is stable in its half-life and mRNA splice selection. Thus, it is conceivable to attribute distinct roles to CB isoforms, whereby CB2 might be involved in long-term synaptic plasticity; and CB1 being critical for homeostatic plasticity.

Unfortunately, in our qPCR or *in situ* hybridization experiments we could not distinguish between all four CB1/CB2 isoforms for technical reasons. The C'-terminus of CB2 is too short for raising specific probes, and the SH3 domain cannot be distinguished between CB1 and CB2. Nevertheless, in overexpression experiments, SH3 domain-containing isoforms always showed some differences compared to the SH3 domain-lacking isoforms, which were often opposite between CB1 and CB2. For example, the half-life of CB is longest for $CB2_{SH3-}$ and shortest for $CB1_{SH3-}$, thus a specific function to the SH3 domain could not be identified. It is generally considered that the SH3 domain exerts an auto-inhibitory function on CB by interacting with amino acid residues of the PH domain, thus burying the DH

domain inside of the molecule. Upon binding to NLGN2, this inhibition is released and CB can interact with the membrane through phosphoinositol binding and stabilize gephyrin postsynaptically by forming a holocomplex with NLGN2 and CB. Through the release of the SH3 domain a structural change occurs, opening CB overall structure, which enhances gephyrin clustering activities (Soykan et al., 2014). The binding of CB to NLGN2 was shown to be critical for postsynaptic assembly at perisomatic GABAergic synapses, since in NLGN2-KO mice gephyrin clustering is lost, especially in perisomatic regions. Furthermore in heterologous systems overexpression of NLGN2 together with gephyrin is not sufficient to form gephyrin clusters but requires also the presence of CB (Poulopoulos et al., 2009). These studies were only performed with CB2 and data about CB1 are missing. Our results suggest, however, that the C'-terminus unique to each isoform also contributes to regulating CB conformation and function. In two crystallographic studies of CB (Xiang et al., 2006; Soykan et al., 2014), the C'-terminus was truncated for stability reasons, but it was proposed that since the C'-terminus of CB2 is positively charged, it could also interact with the negatively charged membrane. This would imply that CB2 can be better stabilized at the membrane. The membrane-anchoring of CB can be even further enhanced by the binding to Cdc42, which has an open conformation upon CB binding and can also interact with the membrane (Xiang et al., 2006). Thus, to better distinguish between CB isoforms, the interaction between the C'-terminus and the SH3 domain needs to be analyzed further.

The interaction between a coiled-coil domain and the SH3 domain was shown in Bcr-Abl to exert auto-inhibition and be important for intramolecular post-translational modification (Smith et al., 2003). Such an interaction could also be important for CB, where we showed that the mutations of lysine residues in the C'-terminus had a different effect on CB protein stability, dependent on the presence/absence of SH3 domain. The results from the lysine mutation analysis uncovered other posttranslational modification, like phosphorylation or hydroxylation, to affect ubiquitination. CB has been shown to directly interact with Smurf1, an E3 ligase involved in SMAD signaling and affecting cell polarity by interacting with RhoA, leading to an activation of Cdc42 and Rac1 (Yamaguchi et al., 2008). Since the binding of Smurf1 was mapped to the PH domain, it is too far away

to promote ubiquitination at the lysines in the C'terminus. However, since ubiquitination is often dependent on post-translational modification, the analysis of these sites and possible enzymes effecting these modifications would be of great relevance to further distinguish the regulation between CB isoforms.

An important finding in the protein stability experiment was that the gephyrin clustering is directly correlated with the stability of CB, since disrupting ubiquitination in CB1 increased gephyrin cluster size in cultured primary neurons and CB2, which has a longer half-life, promotes the formation of larger gephyrin clusters than CB1. This underlines the role of CB to stabilize gephyrin at GABAergic synapses. A recent study demonstrated, that CB is needed for Cdk5 dependent phosphorylation of gephyrin at Ser270 (Kuhse et al., 2012). We showed earlier, that phosphorylation of gephyrin at Ser270 leads to calpain-dependent degradation (Tyagarajan et al., 2011b), thus CB stabilizes gephyrin at the GABAergic synapse not only by promoting NLGN2-gephyrin binding (Papadopoulos and Soykan, 2011), but in addition CB is also involved in activity-dependent post-translational modification of gephyrin itself.

GABAergic synapse are involved in the regulation of dendritic development

Altered dendritic arborization and spine maturation has been reported in many mental disorders, like schizophrenia, autism spectrum disorders, or anxiety (Bergami et al., 2008; Hutsler and Zhang, 2010; Ren et al., 2014). The pathogenesis of these disorders is manifolds, with a variety of mechanisms involved at different time points during life. In relation to this thesis, proteomic studies revealed that besides defects in RhoGTPases and their signaling cascades, altered $GABA_AR$ surface expression is also associated with these disorders (Lionel et al., 2013; Chen et al., 2014).

In our study of adult neurogenesis, we show the importance of CB and its tight regulation by GABAergic synapses for dendritic arborization, thus possibly altering the oval excitability of the neuron. These findings provide a possible link between these two sides of the disorders. This could also go in line with a study showing that disturbed GABAergic transmission during early development increases the vulnerability for anxiety disorder or major depression in adulthood (Shen et al.,

2012). The role of small Rho GTPases for neuronal development is well established and their activation is temporally regulated, since RhoA is involved in proliferation and differentiation of NPC, while Cdc42 and Rac1 are important for dendritic development and spine morphology in a stage specific manner: Cdc42 is involved in early stages, whereas Rac1 in in later stages and in mature neurons (Vadodaria et al., 2013). The underling mechanism is not known yet, but one factor could be that when the neurons are around 3 weeks old, the GABAergic synapse density is the highest and is reduced afterward due to pruning. Thus, the regulating power of GABAergic synapses on Cdc42 activity would be highest during these stages. Several signaling cascades act on a neuron at any given time and it's an open question which factors determine when to grow and when to stop. It was shown, for example, that conditional ablation of BDNF results in shorter dendrites and fewer spines (Chan et al., 2008), and effects of antidepressant treatment are, among others, to increase BDNF expression and function (Schmidt and Duman, 2007). Furthermore, prolonged BDNF treatment of cultured hippocampal neurons increase gephyrin cluster density (González, 2014).

Another signaling protein with reported effects at GABAergic, as well as glutamatergic, synapses is GSKβ. In a recent study, it was shown in organotypic hippocampal slice cultures that dendritic shrinkage arises from reduced $GABA_AR$ surface expression mediated by phosphorylation of Ser270 on gephyrin (Rui et al., 2013). The mechanism for this dendritic shrinkage was mapped to GSKβ activation, which selectively affected GABAergic synapses. The activation of GSKβ results in the phosphorylation of gephyrin at Ser270, which leads to calpain-dependent cleavage and consequently degradation of gephyrin, reducing the number of gephyrin and $GABA_AR$ clusters (Tyagarajan and Fritschy, 2014). The degradation of gephyrin could lead to an increase in unbound CB, which then interacts with Cdc42, sequestering it away from other Cdc42-activating GEFs, leading to reduced dendritic length. In an activity-dependent manner, the splicing of CB1 isoforms is unregulated and newly produced CB could bring gephyrin back to the synapse, ensuring that the reduction of $GABA_AR$ and the resulting hyper- excitability of the neurons is not long-lasting. Furthermore, in the *in vitro* study, we postulated a role for CB1 in GABAergic synapse located in spines, where formation of a complex made of gephyrin, CB and

Cdc42 could be favored, placing Cdc42 in the vicinity of activating GEFs, resulting in single spine increase, as reported by Murakoshi upon CaMKII activation (Murakoshi et al., 2011). The dependency of CB on gephyrin to act on Cdc42 would provide a link between inhibitory and excitatory synapse to regulate homeostatic balance in the neurons.

It would be of great interest to study if the distribution of CB is altered in gephyrin-KO mice to further confirm the dependency of CB for proper function. Since gephyrin-KO mice die shortly after birth, these experiments would require conditional gene deletion. A gene expression assay of gephyrin-KO and WT mice showed no change in CB transcripts; interestingly EFA6 and Frabin, two other GEFs, were down-regulated (PhD Thesis Felix Weltzien, 2010, http://publikationen.ub.uni-frankfurt.de/volltexte/2010/8681/). Both are GEFs involved in cytoskeleton rearrangements. EFA6 activates Arf6, which is involved in dendritic branching (Hernández-Deviez et al., 2004); Furthermore, EFA6 and Arf6 are involved in stabilizing dendritic spines (Choi et al., 2006). Frabin is a RhoGEF specific for Cdc42 (Nakanishi and Takai, 2008) containing a actin-binding motif and a membrane binding domain, like CB. The results of this screen could not be verified by qPCR and further experiments are needed. If CB expression is not altered in gephyrin-KO mice, CB would be placed again upstream of gephyrin; in addition, according to our hypothesis, CB lacking gephyrin interaction should sequester Cdc42 away, leading to opposite effects than with CB silencing.

To maintain homeostatic balance, fast adaptations of the synapses are important, requiring rapid protein synthesis and degradation. The identification of over 2500 mRNA species in dendrites revealed the importance of dendritic protein synthesis for synaptic function and signaling (Cajigas et al., 2012). Therefore, the identification of CB mRNA in dendrites of cultured neurons provides a potential mechanism for local translation, possibly related to specific subsets of GABAergic synapses. It would be of great relevance for the understanding of the regulation of CB isoforms to investigate the mechanisms of CB mRNA transport and targeting to subcellular compartments. One possible interaction partner could be Staufen (Stau), which was shown to be involved in mRNA transport in dendrites, and thus in synaptic plasticity (Heraud-Farlow and Kiebler, 2014). The two orthologues Stau1 and Stau2 have

different targets in dendrites; both are involved in spine morphogenesis, but Stau2 is implicated with LTD whereas Stau1 regulates L-LTP (Goetze et al., 2006; Vessey et al., 2008), indicating the possibility of targeting the mRNA to specific synapses depending on their activity.

Concluding perspectives

Taken together, the results of the two studies presented here reveal two distinct functions of CB. On one hand, CB regulates gephyrin clustering in an isoform-specific manner, whereby activity-dependent mRNA splicing of CB might modulate synaptic plasticity. On the other hand, CB controls Cdc42 activity for the regulation of cell development and spine formation. Surprisingly, our results suggest that CB largely inhibits Cdc42 activity, unless bound to gephyrin, providing a novel mechanism for spatially and temporally regulated Cdc42 activation (Figure IV).

To further understand the isoform-specific regulation of gephyrin, it will be crucial to further investigate the targeting of CB to different GABAergic synapses and to determine regulatory proteins mediating post-translational modification on CB C'-terminus. Furthermore it would be interesting to elucidate the interaction between the SH3 domain and the C'-terminus and shed more light on the importance of CB structure and its function to cluster gephyrin.

To verify the negative regulation of CB on Cdc42 activation, newborn GCs expressing a dominant negative Cdc42 should be investigated further for migration and gephyrin clustering. Since overexpression of CB in newborn neurons was only studied with $CB1_{SH3-}$, it would be of great interest to study also $CB2_{SH3-}$ to determine whether the interaction with Cdc42 is isoform-specific, and with $CB1_{SH3+}$ to analyze the possible auto-inhibitory mechanism of CB interacting with Cdc42. These experiments would solidify our hypothesis.

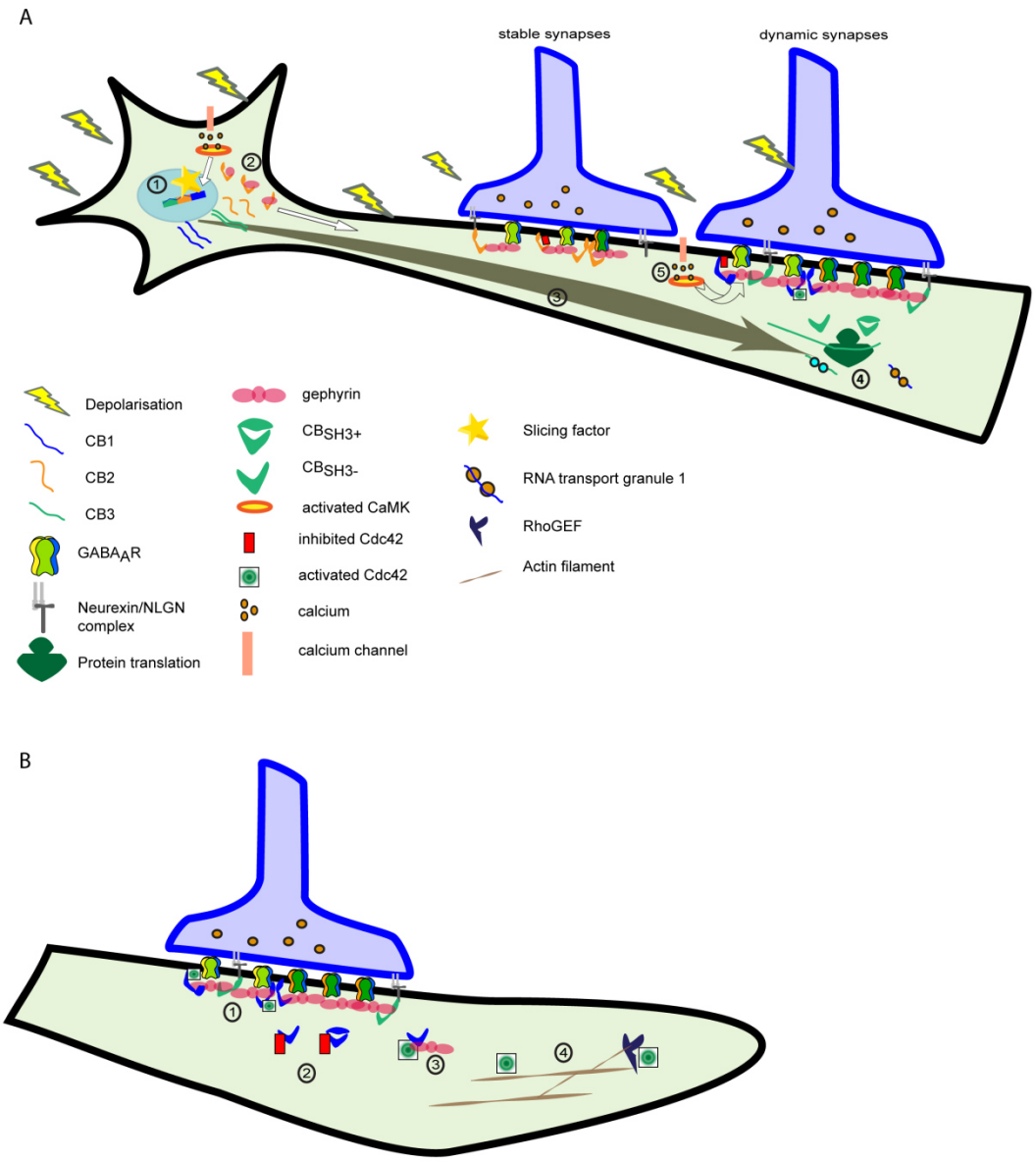

Figure IV: Model explaining the two functions of CB. A.: Possible role of CB isoforms for plasticity changes at GABAergic synapse. (1) Activity-dependent and CaMK-dependent splicing of CB mRNA isoforms. **(2)** CB2 somatic translation stabilizes gephyrin and transports it to stable GABAergic synapses. **(3)** Dendritic transport of CB1 and CB3 mRNAs after activity dependent splicing changes for dendritic translation. **(4)** Local protein synthesis of CB1 and CB3 splice isoforms promote gephyrin clustering at dynamic GABAergic synapses. **(5)** Activity-dependent and Ca^{2+} dependent mechanisms facilitate Ub mediated CB1/CB3 protein turnover at dynamic synapses to promote gephyrin cluster loss and $GABA_AR$ internalization. **B. Cdc42 activity regulation through CB. (1)** Cdc42 located at the GABAergic synapse, interacting with CB and gephyrin is activated to modulate gephyrin cluster size. **(2)** Cdc42 bound to CB is inactive and sequester away from other RhoGEFs. **(3)** Complex of CB and gephyrin activates Cdc42. **(4)** Free Cdc42 can be activated by RhoGEFs and induce actin remodeling for dendritic outgrowth.

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ABBREVIATIONS

ACSF	Artifical cerebrospinal fluid
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
BZD	Benzodiazepine
CaMK	Calmodulin-dependent protein kinase
CB	Collybistin
CCK	Cholecystokinin
Cdc42	Cell division control protein 42 homolog
Cdk	Cycline-dependent kinase
CNS	Central nervous system
DG	Dentate Gyrus
DH	dbl-homology
dpi	Days post-injection
DIV	Days *in vitro*
DTT	Dithiothreitol
E/I	Excitation and inhibition
eIPSC	evoked inhibitory postsynaptic current
ERK	Extracellular regulated kinase
FBS	Fetal bovine serum
FGS	Fetal goat serum
GABA	γ-aminobutyric acid
$GABA_AR$	$GABA_A$ Receptor
$GABA_BR$	$GABA_B$ Receptor
GABAT	GABA transporter
GAD	Glutamate decarboxylase
GC	Granule cell
GCL	Granule cell layer
GDP	guanosine diphosphate
GEF	Guanine nucleotide exchange factor
eGFP	Enhanced Green fluorescent protein
GlyR	Glycine receptor
GSK3β	Glycogen synthase kinase 3β
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HEK	Human Embryonic Kidney
i.p.	Intraperitoneal injection
IPSP	inhibitory postsynaptic potential
KCC2	K^+-Cl^- co-transporter
KO	Knockout
LTD	Long-trm depression
LTP	Long-term potentiation
mIPSC	miniature inhibitory postsynaptic current
MoCo	Molybdenum cofactor
NCAM	Neural cell adhesion molecule
NGF	Neural growth factor
NGS	Normal goat serum
NKCC1	Na^+-K^+-$2Cl^-$ co-transporter
NLGN	Neuroligin
NPC	Neural progenitor cell
OB	Olfactory bulb
O-LM	oriens-lacunosum moleculare

PCR	polymerase chain reaction	RV	Retrovirus
PEI	Polyethylenimine	RT	room temperature
PH	Pleckstrin-homology	SE	Status epilepticus
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate	SGZ	Subgranular zone
		SH3	scr-homology 3
PSD	Postsynaptic density	SVZ	Subventricular zone
PV	Parvalbumin	vGAT	vesicular GABA transporter
Rac	Ras-related C3	vGLUT	vesicular glutamate transporter
Rho	ras homologue		
RMS	Rostral migratory stream	VZ	Ventricular zone

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PUBLICATIONS

ORIGINAL ARTICLES

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ABSTRACTS

C. de Groot, M. I. Abdul and S. K. Tyagarajan (2012)
Collybistin: The interplay of different splice variants to regulate GABAergic postsynaptic proteins
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C. de Groot and S. K. Tyagarajan (2012)
Collybistin: The interplay of different splice variants to regulate GABAergic postsynaptic proteins
Swiss Society for Neuroscience, SSN Annual Meeting, Zurich, Switzerland

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Collybistin: The interplay of different splice variants to regulate GABAergic postsynaptic proteins
8th Forum of European Neuroscience (FENS), Barcelona, Spain

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Activity-dependent mechanisms and ubiquitin pathway determine collybistin function at GABAergic synapses
Mechanisms of GABAergic synaptic plasticity, Chexbres, Switzerland

C. de Groot, A. Azzi, M. I. Abdul, S. A. Brown and S. K. Tyagarajan (2013)
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Swiss Society for Neuroscience, SSN Annual Meeting, Bern, Switzerland

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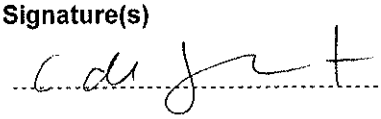
Zurich, 03.11.2014	

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